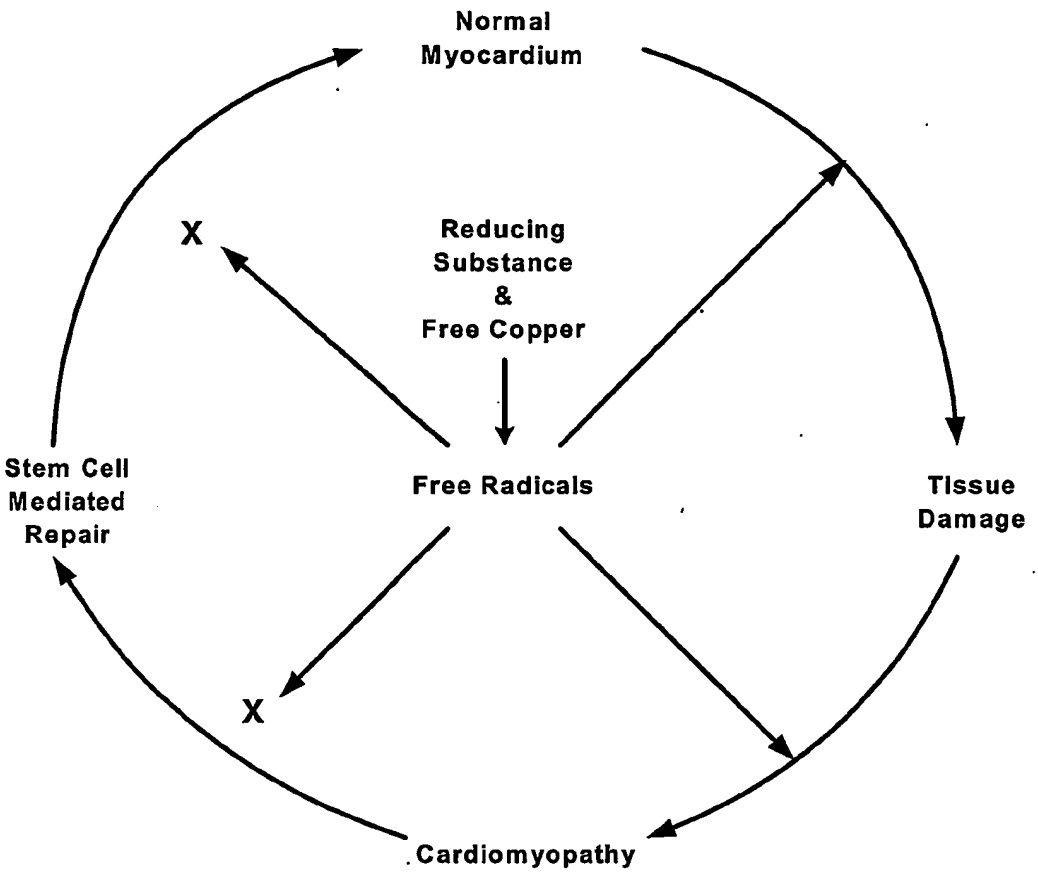

Normal
Myocardium
X
Reducing
Substance
&
Free Copper
Stem Cell
Mediated
Repair
Free Radicals
Tissue
Damage
X
Cardiomyopathy

FIGURE 1

Hypothesis

I. **Glyco-oxidation product ion (AGE* formation)**

Lipid or Protein + glucose → {Protein - AGE
{Lipid - AGE

Cu-AGE Protein or Cu-AGE Lipid → Protein + O_2^-

2. **Superoxide dismutase**

$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$

3. **Haber-Weiss Reaction** (Copper salt catalyst)

$H_2O_2 + 2O_2^- \rightarrow O_2 + OH\bullet + OH^-$

*AGE = Advance Glycation Endproduct

FIGURE 2

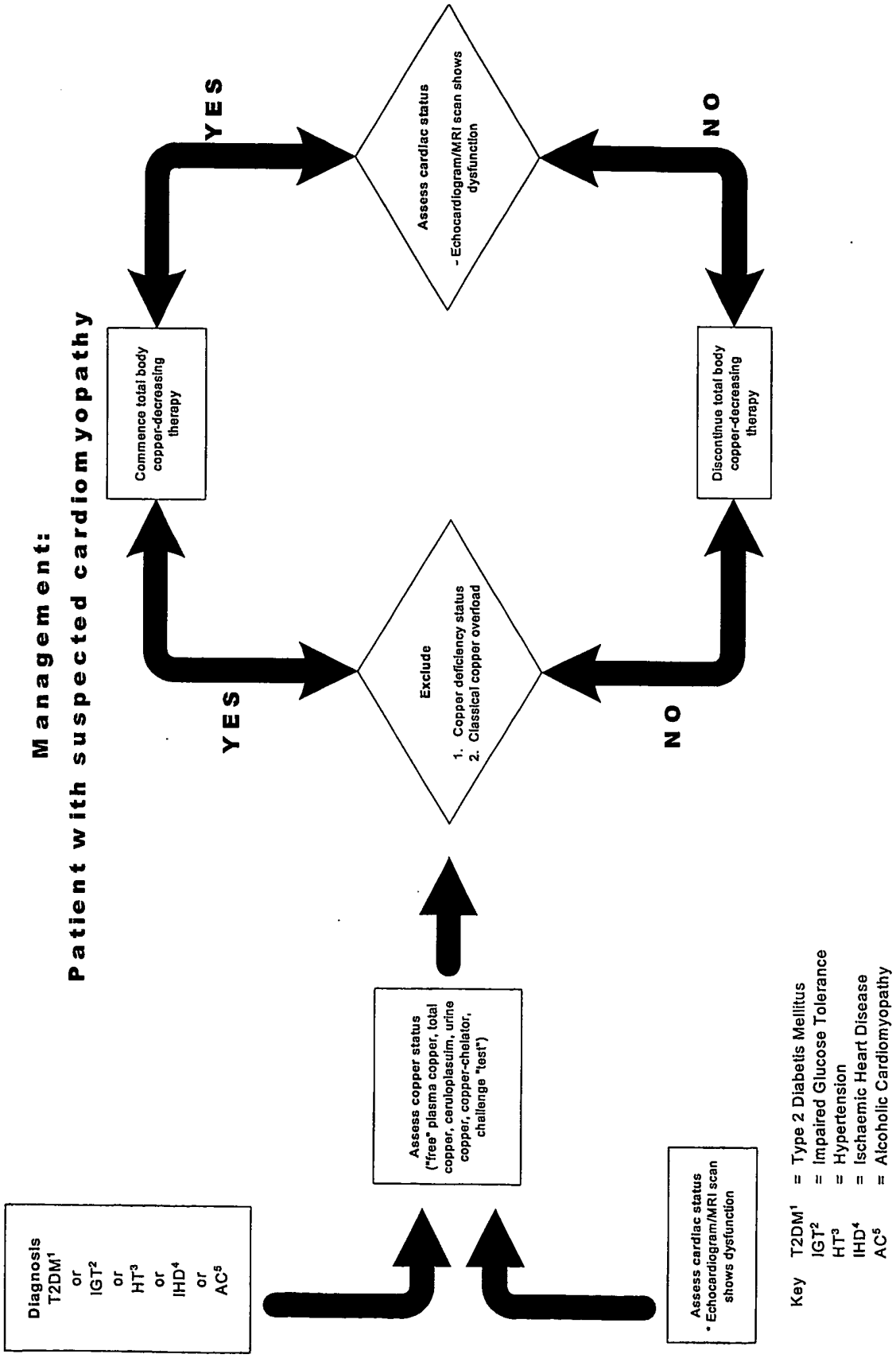

Management:
Patient with suspected cardiomyopathy
Diagnosis
T2DM1
or
IGT2
or
HT3
or
IHD4
or
AC5
Assess cardiac status
• Echocardiogram/MRI scan shows dysfunction
Assess copper status
("free" plasma copper, total copper, ceruloplasuim, urine copper, copper-chelator, challenge "test")
Exclude
1. Copper deficiency status
2. Classical copper overload
YES
NO
Assess cardiac status
- Echocardiogram/MRI scan shows dysfunction
YES
NO
Commence total body copper-decreasing therapy
Discontinue total body copper-decreasing therapy
Key T2DM1 = Type 2 Diabetis Mellitus
IGT2 = Impaired Glucose Tolerance
HT3 = Hypertension
IHD4 = Ischaemic Heart Disease
AC5 = Alcoholic Cardiomyopathy

FIGURE 3

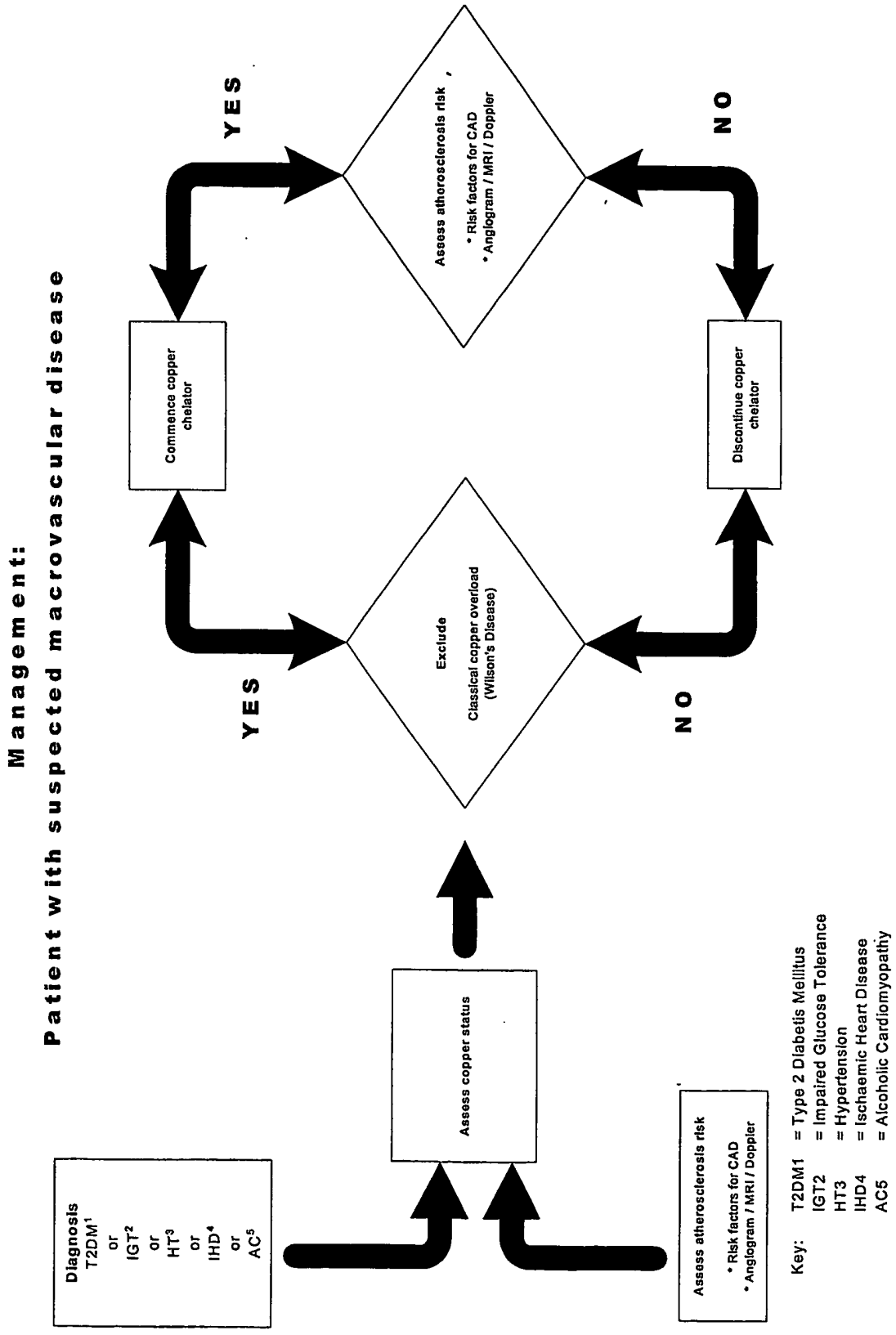

Management:
Patient with suspected macrovascular disease
Diagnosis
T2DM1
or
IGT2
or
HT3
or
IHD4
or
AC5
Assess copper status
Assess atherosclerosis risk
* Risk factors for CAD
* Anglogram / MRI / Doppler
Exclude
Classical copper overload
(Wilson's Disease)
YES
NO
Assess atherosclerosis risk
* Risk factors for CAD
* Anglogram / MRI / Doppler
YES
NO
Commence copper chelator
Discontinue copper chelator
Key: T2DM1 = Type 2 Diabetis Mellitus
IGT2 = Impaired Glucose Tolerance
HT3 = Hypertension
IHD4 = Ischaemic Heart Disease
AC5 = Alcoholic Cardiomyopathy

FIGURE 4

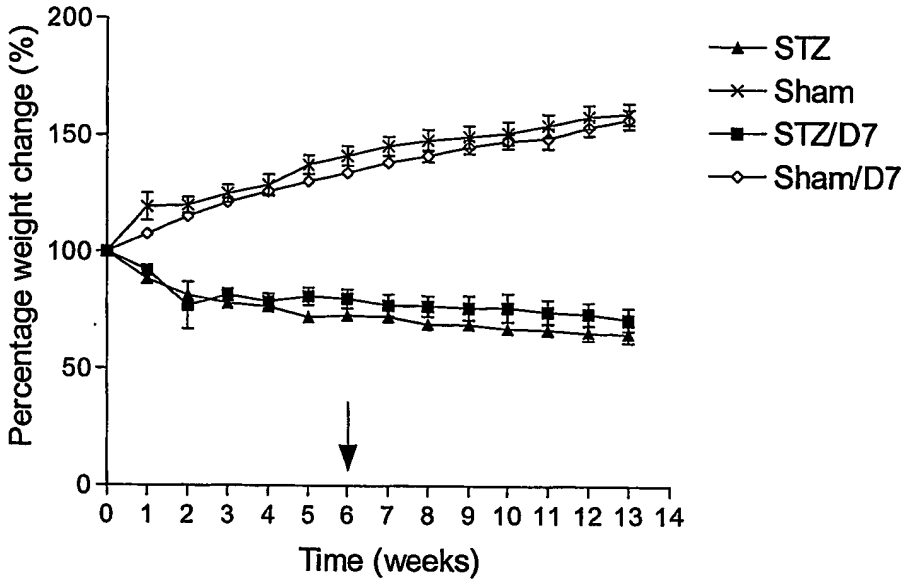

Percentage weight change (%)
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Time (weeks)
STZ
Sham
STZ/D7
Sham/D7

FIGURE 5

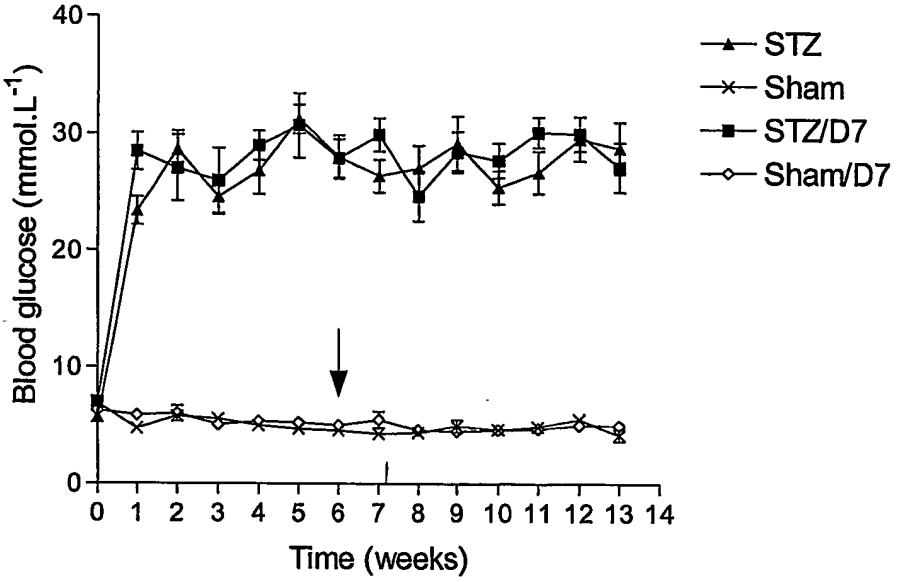
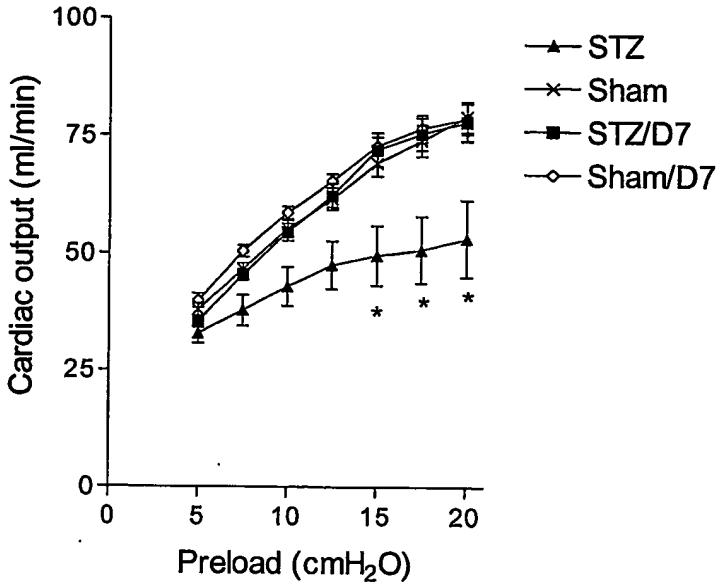
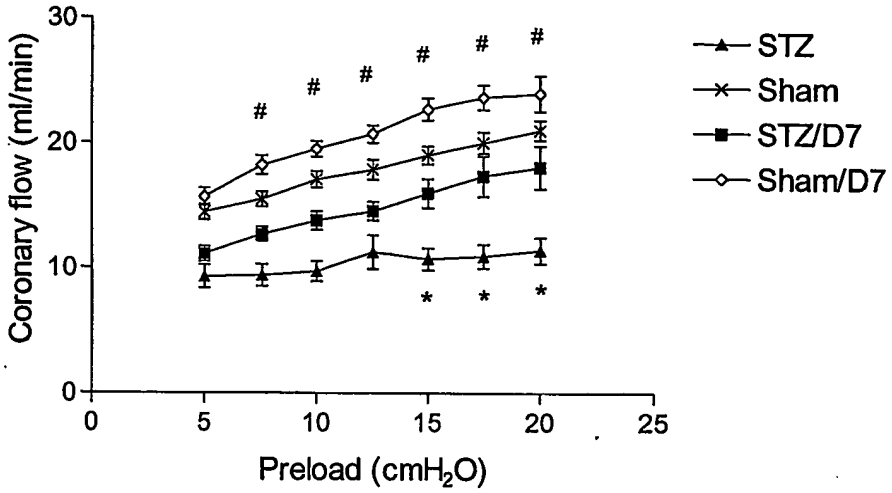

Blood glucose (mmol.L-1)
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Time (weeks)
STZ
Sham
STZ/D7
Sham/D7

FIGURE 6



* p<0.05: STZ v STZ/D7

FIGURE 7



* p<0.05: STZ v STZ/D7, #.p<0.05: STZ/D7 v Sham/D7.

FIGURE 8

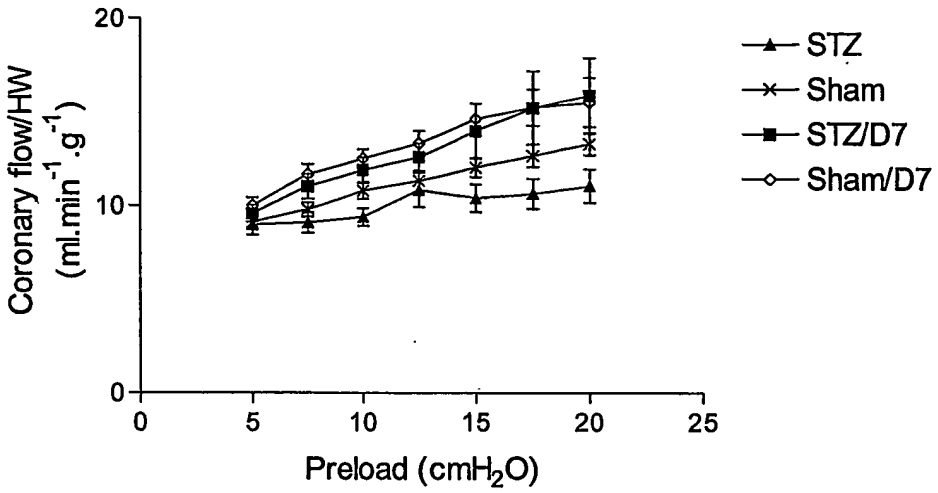
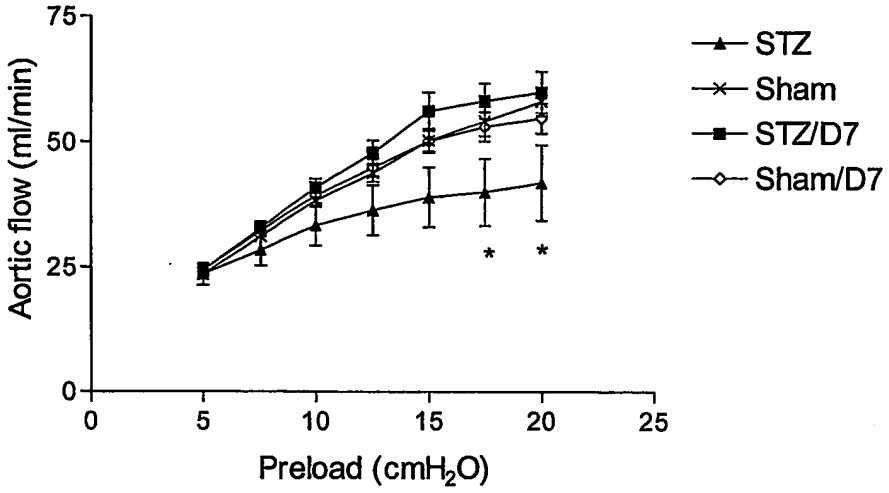
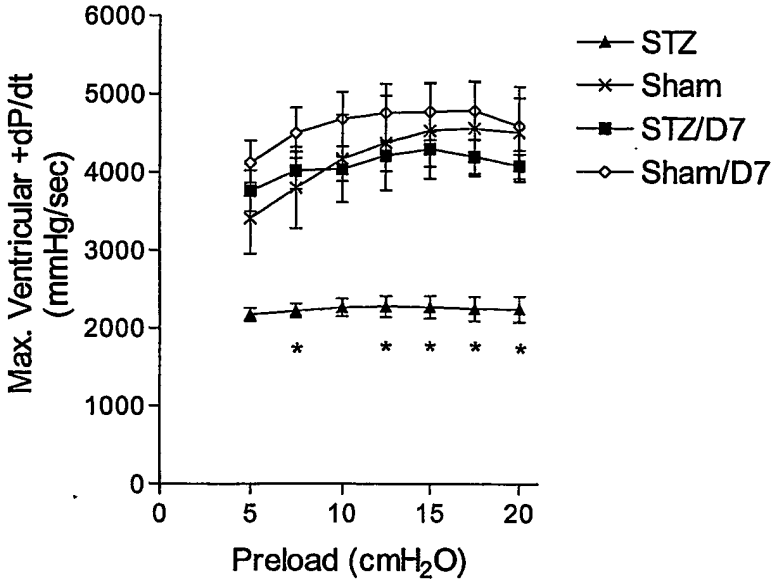

20
10
0
Coronary flow/HW
(ml.min-1.g-1)
0
5
10
15
20
25
Preload (cmH2O)
STZ
Sham
STZ/D7
Sham/D7

FIGURE 9



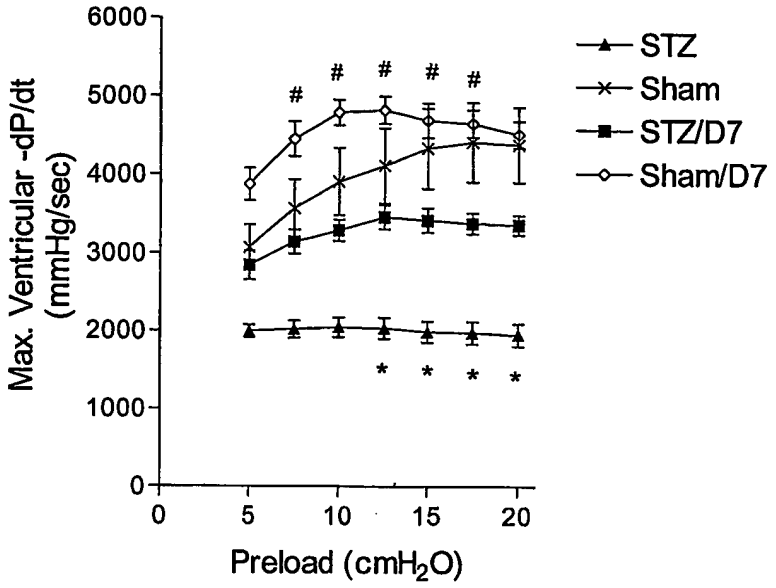
* p<0.05: STZ v STZ/D7

FIGURE 10



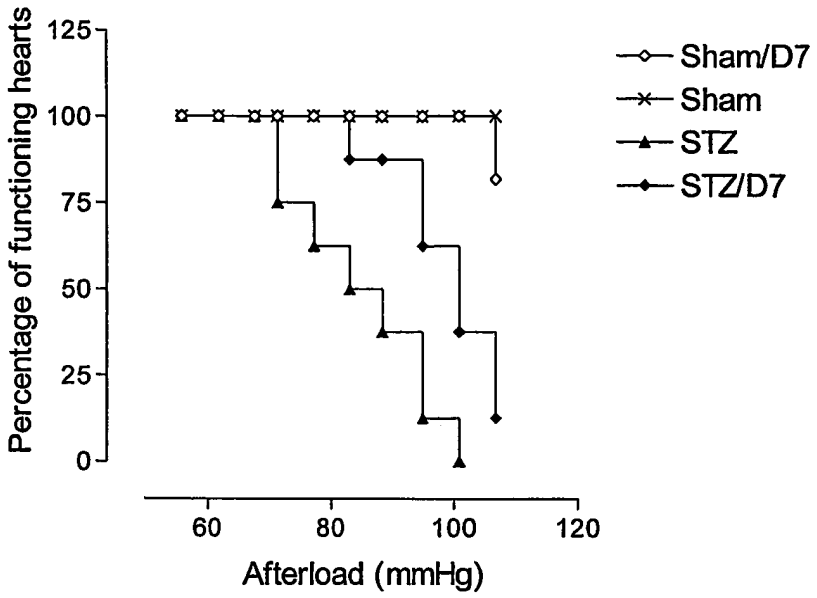
p<0.05: STZ v STZ/D7

FIGURE 11



p<0.05: STZ v STZ/D7, #.p<0.05: STZ/D7 v Sham/D7.

FIGURE 12



Wilcoxon $p<0.05$ for STZ v STZ/D7

FIGURE 13

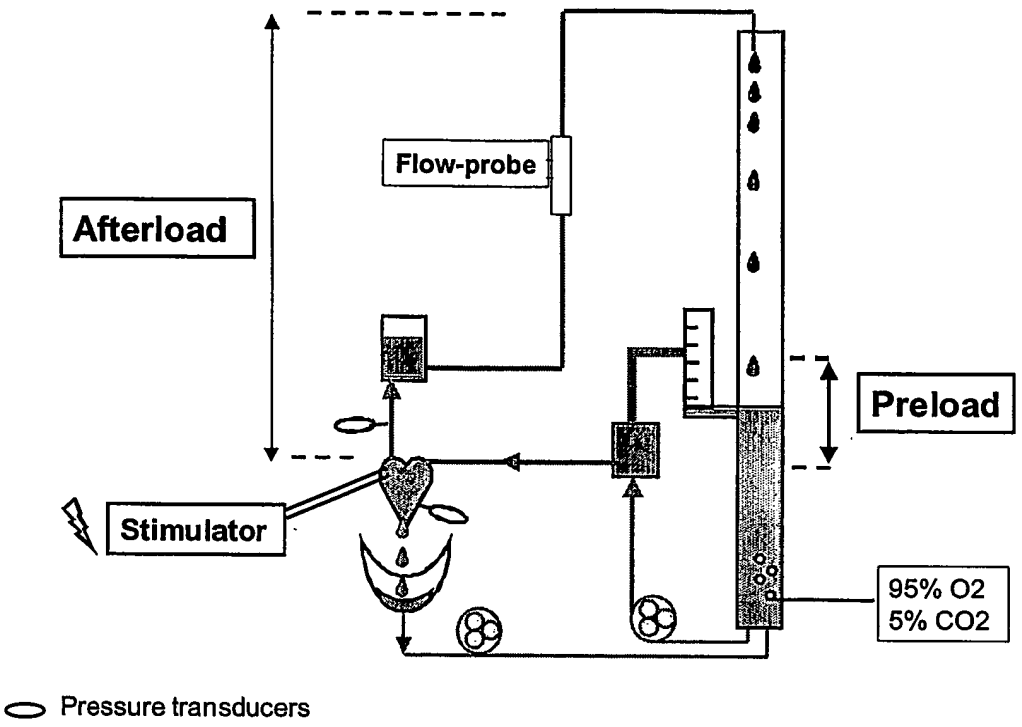

Flow-probe
Afterload
Preload
Stimulator
95% O2
5% CO2
Pressure transducers

FIGURE 14

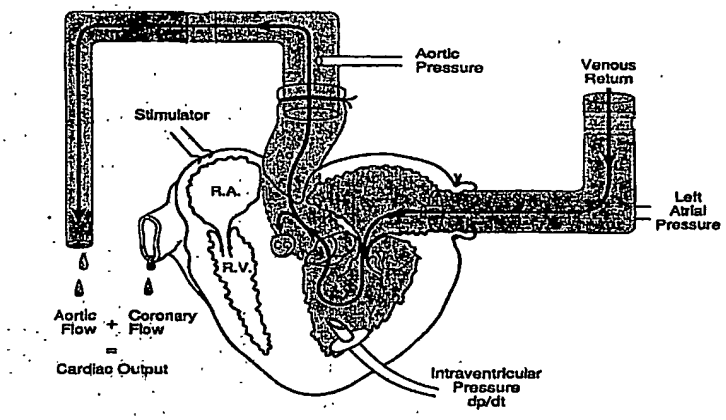

Aortic Pressure
Venous Return
Stimulator
R.A.
Left Atrial Pressure
R.V.
Aortic Flow
+
Coronary Flow
=
Cardiac Output
Intraventricular Pressure dp/dt

FIGURE 15

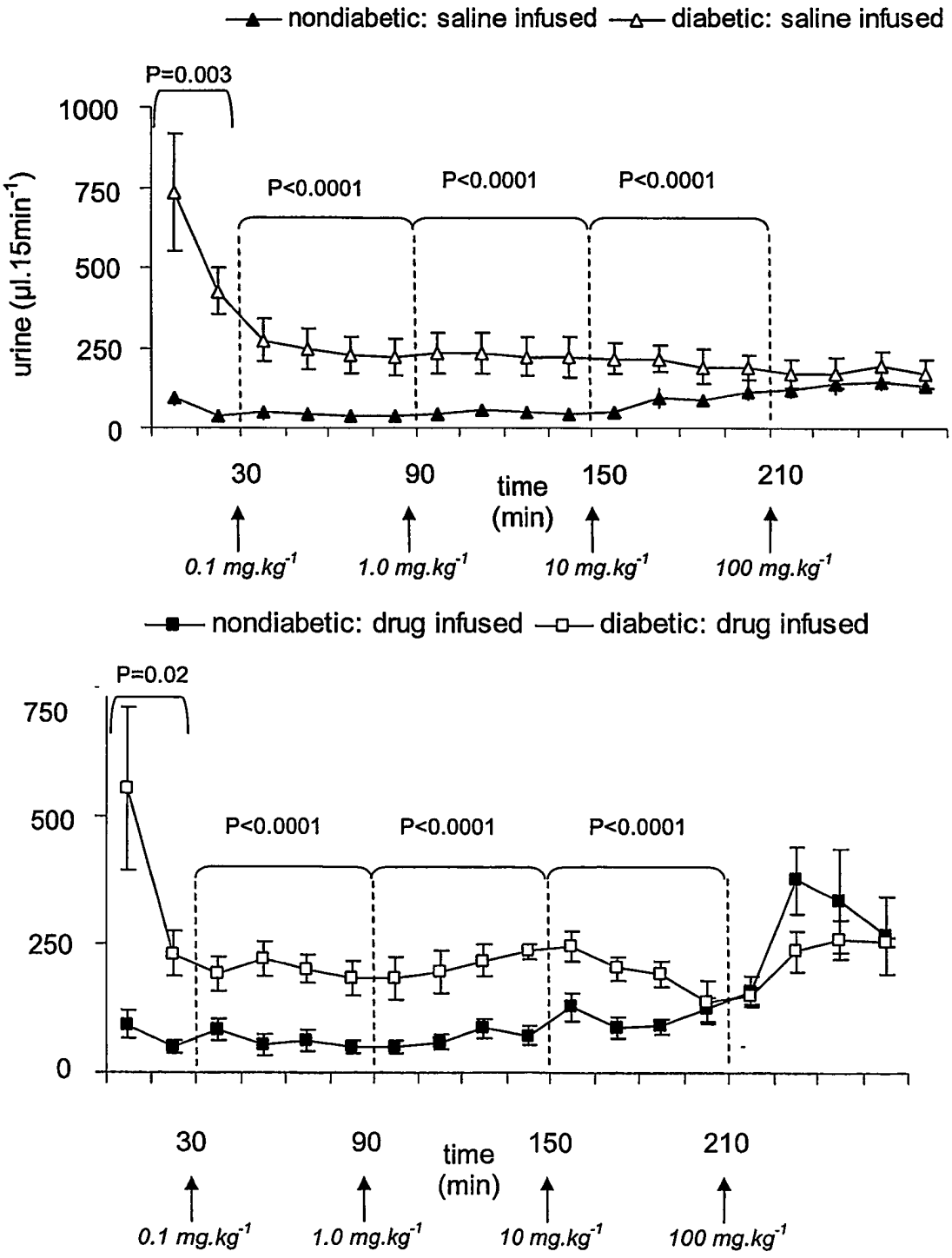

nondiabetic: saline infused
diabetic: saline infused
P=0.003
1000
750
500
250
0
urine (µl.15min-1)
P<0.0001
P<0.0001
P<0.0001
30
90
150
210
time (min)
0.1 mg.kg-1
1.0 mg.kg-1
10 mg.kg-1
100 mg.kg-1
nondiabetic: drug infused
diabetic: drug infused
P=0.02
750
500
250
0
P<0.0001
P<0.0001
P<0.0001
30
90
150
210
time (min)
0.1 mg.kg-1
1.0 mg.kg-1
10 mg.kg-1
100 mg.kg-1

FIGURE 16

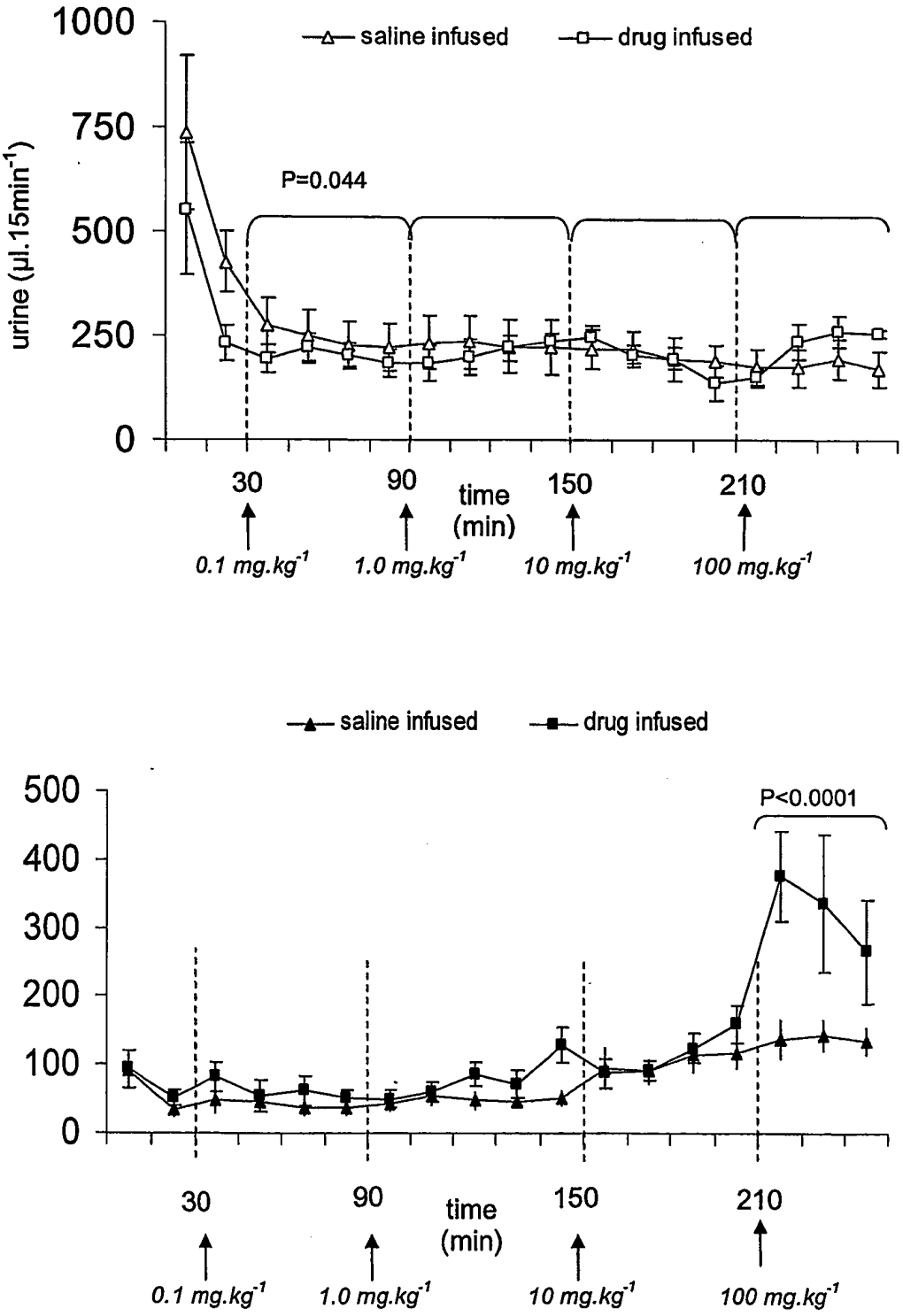

saline infused
drug infused
urine (µl.15min-1)
1000
750
500
250
0
P=0.044
30
90
150
210
time (min)
0.1 mg.kg-1
1.0 mg.kg-1
10 mg.kg-1
100 mg.kg-1
saline infused
drug infused
500
400
300
200
100
0
P<0.0001
30
90
150
210
time (min)
0.1 mg.kg-1
1.0 mg.kg-1
10 mg.kg-1
100 mg.kg-1

FIGURE 17

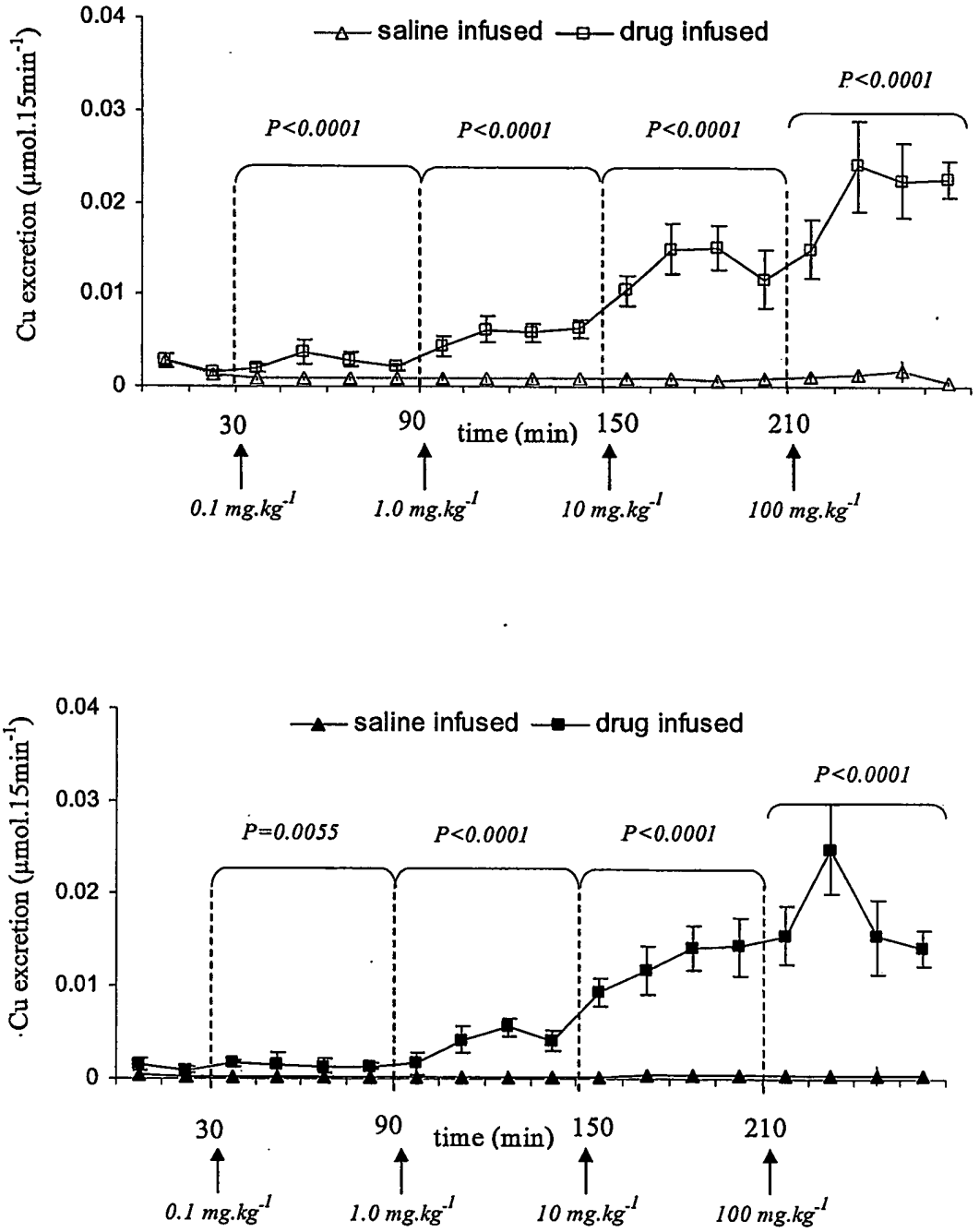

saline infused
drug infused
Cu excretion (µmol.15min-1)
0.04
0.03
0.02
0.01
0
P<0.0001
P<0.0001
P<0.0001
P<0.0001
30
90
time (min)
150
210
0.1 mg.kg-1
1.0 mg.kg-1
10 mg.kg-1
100 mg.kg-1
saline infused
drug infused
Cu excretion (µmol.15min-1)
0.04
0.03
0.02
0.01
0
P=0.0055
P<0.0001
P<0.0001
P<0.0001
30
90
time (min)
150
210
0.1 mg.kg-1
1.0 mg.kg-1
10 mg.kg-1
100 mg.kg-1

FIGURE 18

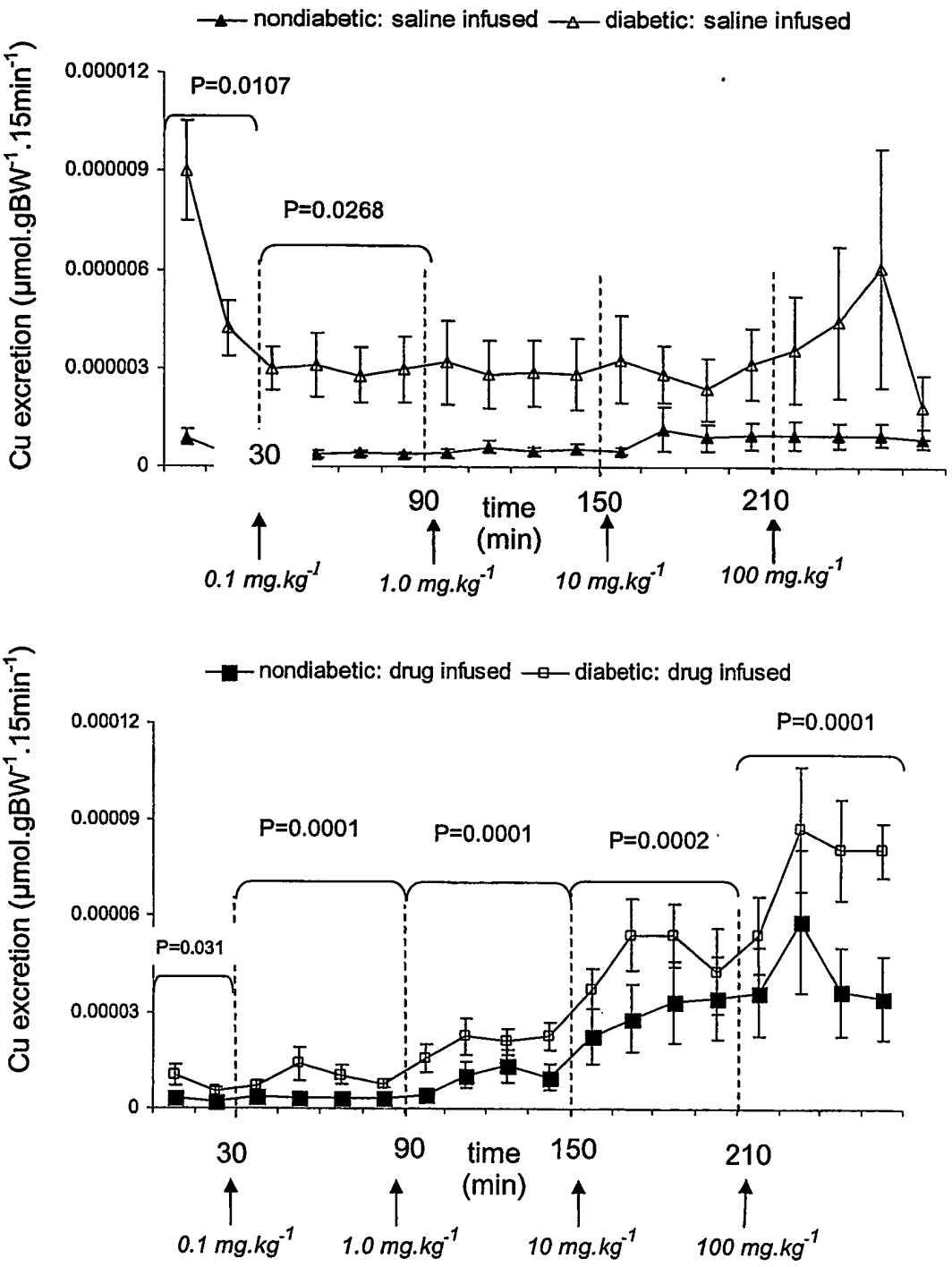

nondiabetic: saline infused
diabetic: saline infused
Cu excretion (µmol.gBW-1.15min-1)
0.000012
0.000009
0.000006
0.000003
0
P=0.0107
P=0.0268
30
90
time (min)
150
210
0.1 mg.kg-1
1.0 mg.kg-1
10 mg.kg-1
100 mg.kg-1
nondiabetic: drug infused
diabetic: drug infused
Cu excretion (µmol.gBW-1.15min-1)
0.00012
0.00009
0.00006
0.00003
0
P=0.0001
P=0.031
P=0.0001
P=0.0002
P=0.0001
30
90
time (min)
150
210
0.1 mg.kg-1
1.0 mg.kg-1
10 mg.kg-1
100 mg.kg-1

FIGURE 19

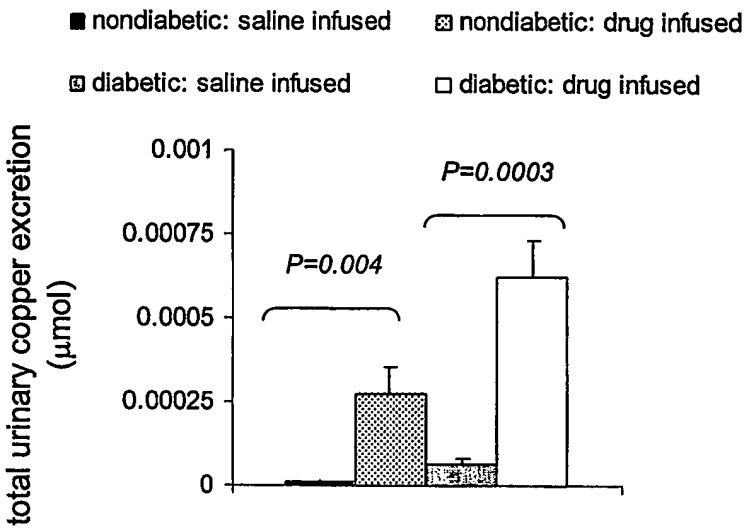

nondiabetic: saline infused
nondiabetic: drug infused
diabetic: saline infused
diabetic: drug infused
total urinary copper excretion (µmol)
0.001
0.00075
0.0005
0.00025
0
P=0.004
P=0.0003

FIGURE 20

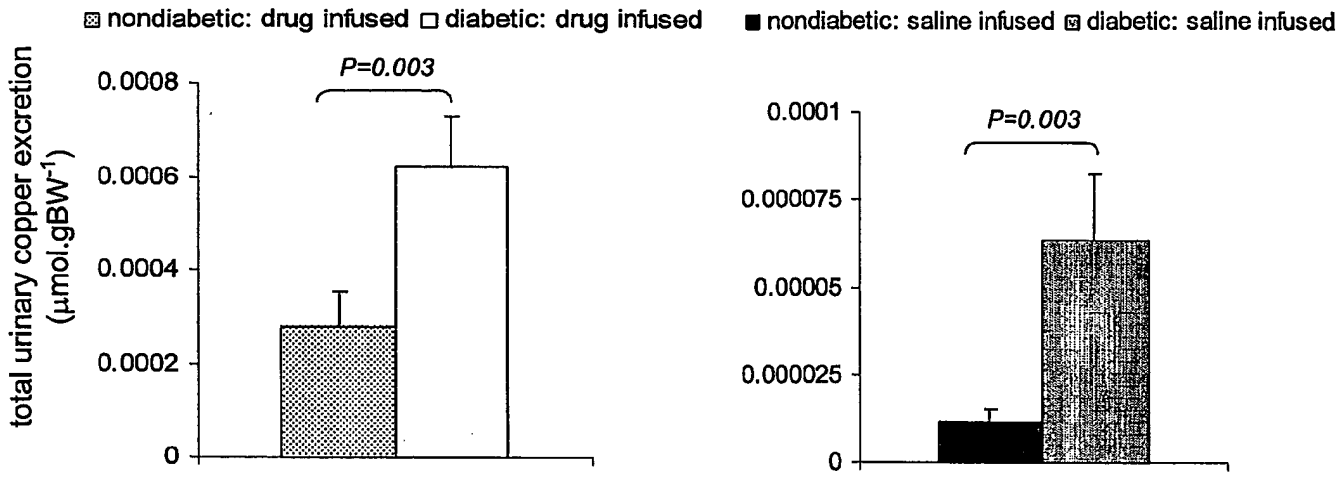

nondiabetic: drug infused
diabetic: drug infused
nondiabetic: saline infused
diabetic: saline infused
total urinary copper excretion (µmol.gBW-1)
P=0.003
0.0008
0.0006
0.0004
0.0002
0
P=0.003
0.0001
0.000075
0.00005
0.000025
0

FIGURE 21

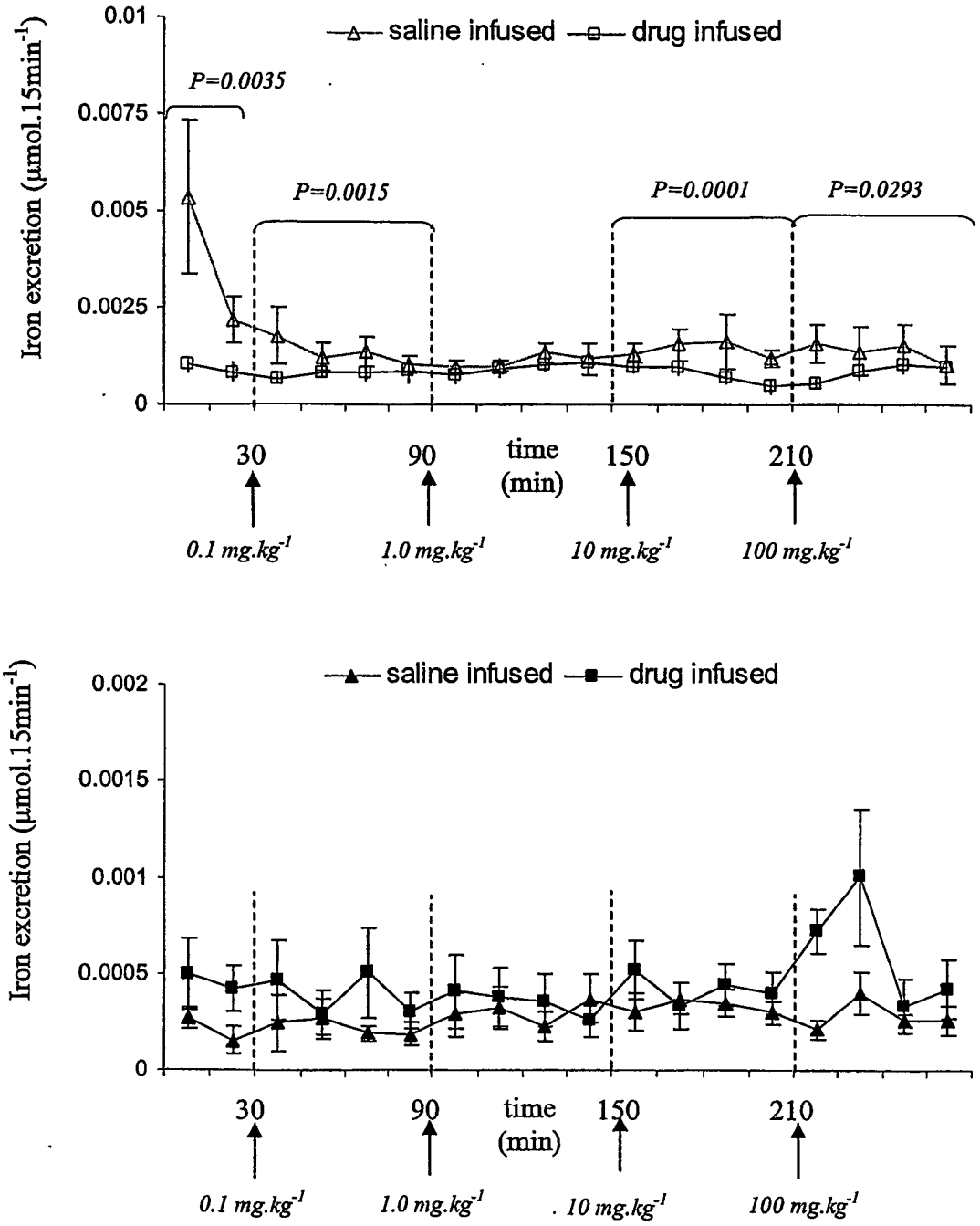

saline infused
drug infused
Iron excretion (µmol.15min-1)
0.01
0.0075
0.005
0.0025
0
P=0.0035
P=0.0015
P=0.0001
P=0.0293
30
90
time (min)
150
210
0.1 mg.kg-1
1.0 mg.kg-1
10 mg.kg-1
100 mg.kg-1
saline infused
drug infused
Iron excretion (µmol.15min-1)
0.002
0.0015
0.001
0.0005
0
30
90
time (min)
150
210
0.1 mg.kg-1
1.0 mg.kg-1
10 mg.kg-1
100 mg.kg-1

FIGURE 22

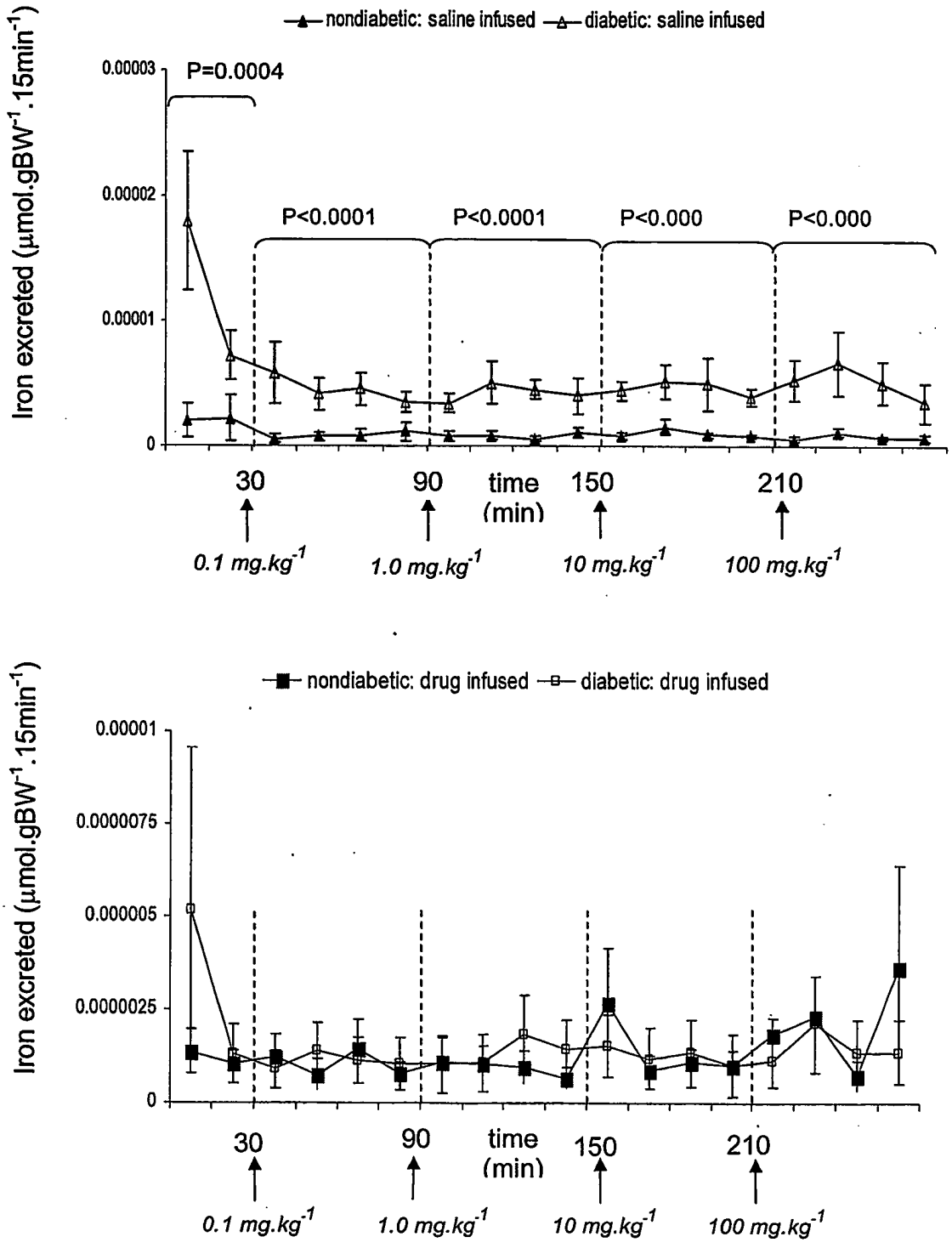

nondiabetic: saline infused
diabetic: saline infused
Iron excreted (µmol.gBW-1.15min-1)
0.00003
0.00002
0.00001
0
P=0.0004
P<0.0001
P<0.0001
P<0.000
P<0.000
30
90
time (min)
150
210
0.1 mg.kg-1
1.0 mg.kg-1
10 mg.kg-1
100 mg.kg-1
nondiabetic: drug infused
diabetic: drug infused
Iron excreted (µmol.gBW-1.15min-1)
0.00001
0.0000075
0.000005
0.0000025
0
30
90
time (min)
150
210
0.1 mg.kg-1
1.0 mg.kg-1
10 mg.kg-1
100 mg.kg-1

FIGURE 23

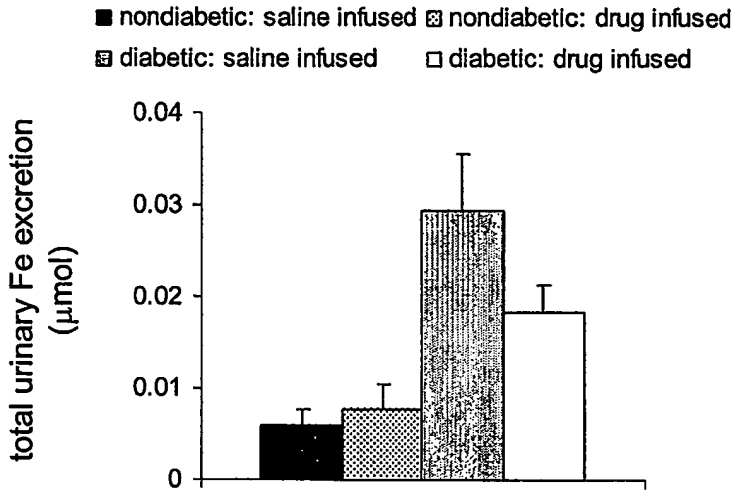

nondiabetic: saline infused
nondiabetic: drug infused
diabetic: saline infused
diabetic: drug infused
total urinary Fe excretion (µmol)
0.04
0.03
0.02
0.01
0

FIGURE 24

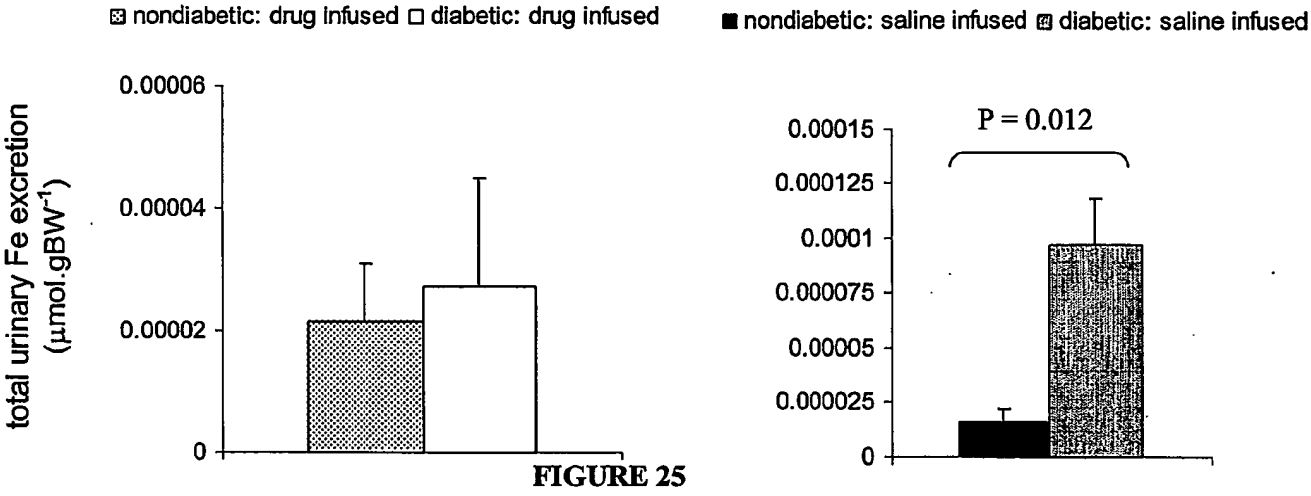

nondiabetic: drug infused
diabetic: drug infused
nondiabetic: saline infused
diabetic: saline infused
total urinary Fe excretion (µmol.gBW-1)
0.00006
0.00004
0.00002
0
P = 0.012
0.00015
0.000125
0.0001
0.000075
0.00005
0.000025
0

FIGURE 25

Cu excretion			Dose level		
Mixed Model Effects	**Baseline**	**0.1 mg.kg^{-1}**	**1.0 mg.kg^{-1}**	**10 mg.kg^{-1}**	**100 mg.kg^{-1}**
Diabetes	$F_{1,24}$ = 18.52	$F_{1,24}$ = 19.82	$F_{1,24}$ = 21.92	$F_{1,24}$ = 9.93	$F_{1,24}$ = 17.82
(normal/diabetic rats)	P = 0.0002	P = 0.0002	P < 0.0001	P < 0.0001	P < 0.0003
Drug	$F_{1,24}$ = 1.73	$F_{1,24}$ = 24.94	$F_{1,24}$ = 78.36	$F_{1,24}$ = 135.36	$F_{1,24}$ = 162.17
(drug/saline)	*NS*	P < 0.0001	P < 0.0001	P < 0.0001	P < 0.0001
Interaction	$F_{1,24}$ = 0.16	$F_{1,24}$ = 3.58	$F_{1,24}$ = 7.16	$F_{1,24}$ = 6.02	$F_{1,24}$ = 12.43
	NS	*NS*	P < 0.0132	P < 0.0218	P < 0.0017
Sampling time (repeated measure)	t_1, t_2	t_1, t_2, t_3, t_4	t_1, t_2, t_3, t_4	t_1, t_2, t_3, t_4	t_1, t_2, t_3, t_4

Fe excretion			Dose level		
Mixed Model Effects	**Baseline**	**0.1 mg.kg^{-1}**	**1.0 mg.kg^{-1}**	**10 mg.kg^{-1}**	**100 mg.kg^{-1}**
Diabetes	$F_{1,23}$ = 12.87	$F_{1,23}$ = 15.82	$F_{1,24}$ = 22.68	$F_{1,24}$ = 14.93	$F_{1,24}$ = 7.35
(normal/diabetic rats)	P = 0.0016	P = 0.0006	P < 0.0001	P = 0.0007	P = 0.0122
Drug	$F_{1,23}$ = 8.6	$F_{1,23}$ = 7.89	$F_{1,24}$ = 12.23	$F_{1,24}$ = 10.91	$F_{1,24}$ = 2.47
(drug/saline)	P = 0.0075	P = 0.01	P < 0.0019	P = 0.003	P = 0.1292
Interaction	$F_{1,23}$ = 12.10	$F_{1,23}$ = 15.06	$F_{1,24}$ = 14.07	$F_{1,24}$ = 17.72	$F_{1,24}$ = 16.76
	P = 0.002	P = 0.0008	P = 0.001	P = 0.0003	P = 0.0004
Sampling time (repeated measure)	t_1, t_2,	t_1, t_2, t_3, t_4	t_1, t_2, t_3, t_4	t_1, t_2, t_3, t_4	t_1, t_2, t_3, t_4

FIGURE 26

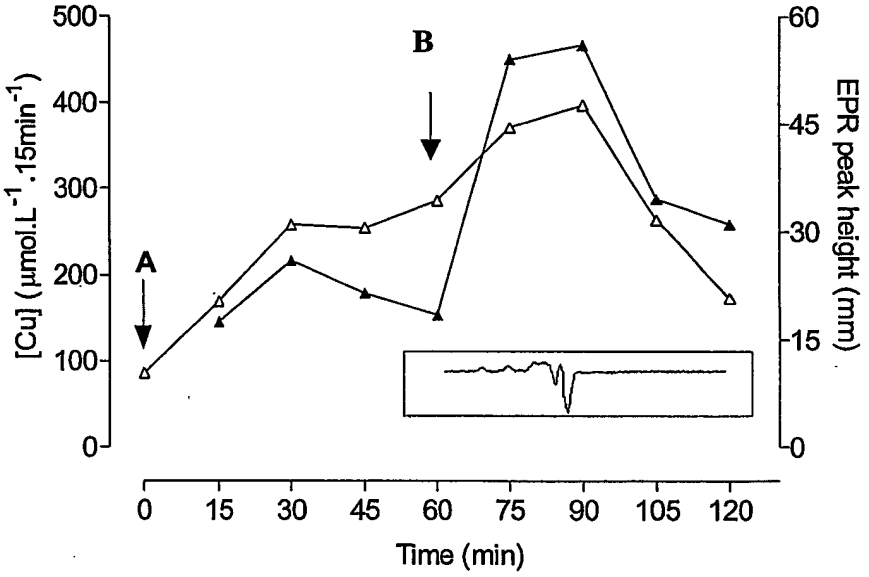

500
400
300
200
100
0
[Cu] (µmol.L-1.15min-1)
60
45
30
15
0
EPR peak height (mm)
A
B
0
15
30
45
60
75
90
105
120
Time (min)

FIGURE 27

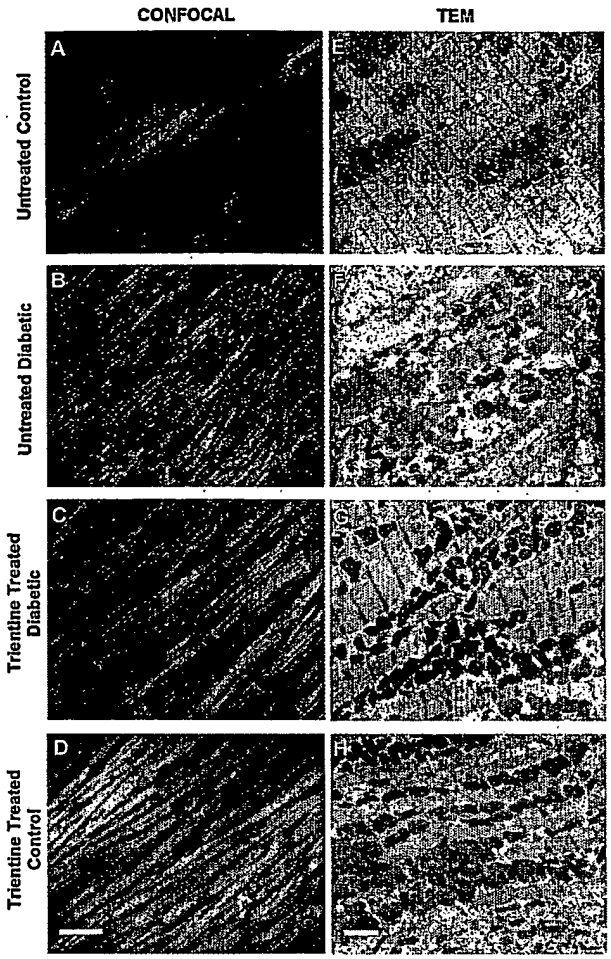

CONFOCAL
TEM
A
E
Untreated Control
B
F
Untreated Diabetic
C
G
Trientine Treated Diabetic
D
H
Trientine Treated Control

FIGURE 28

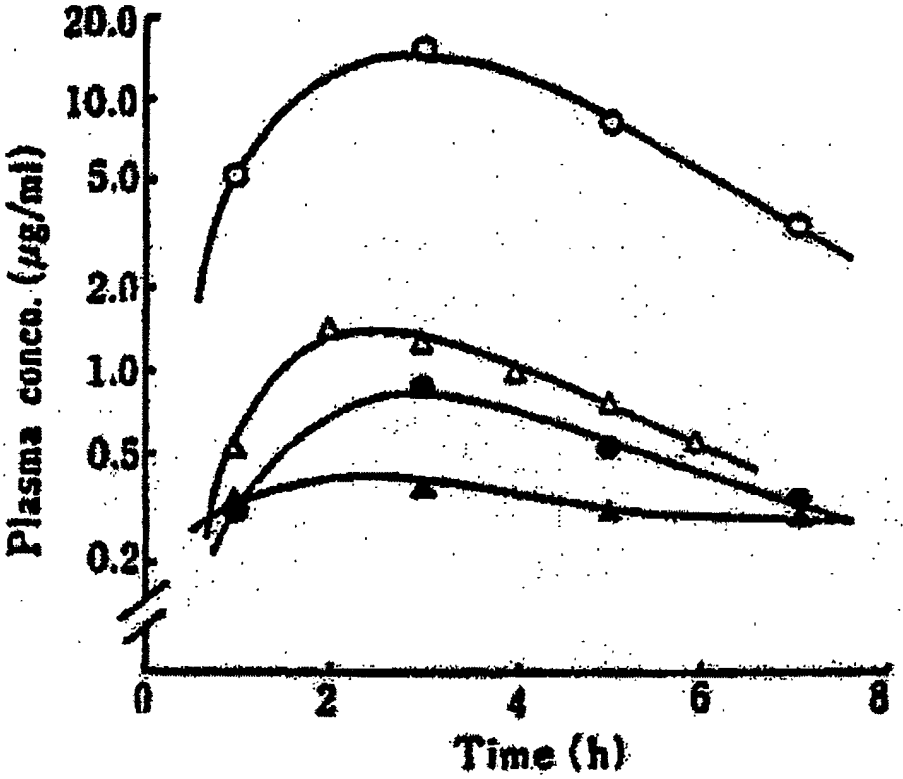


FIGURE 29

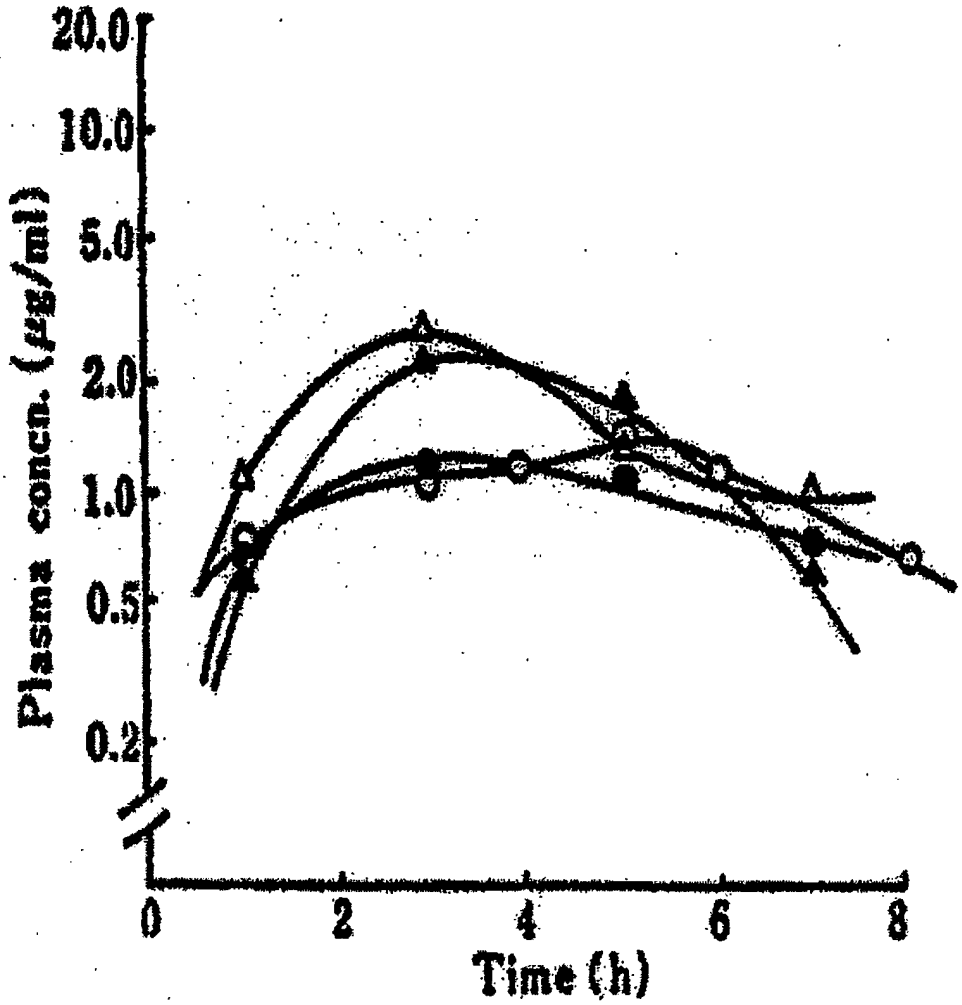


FIGURE 30

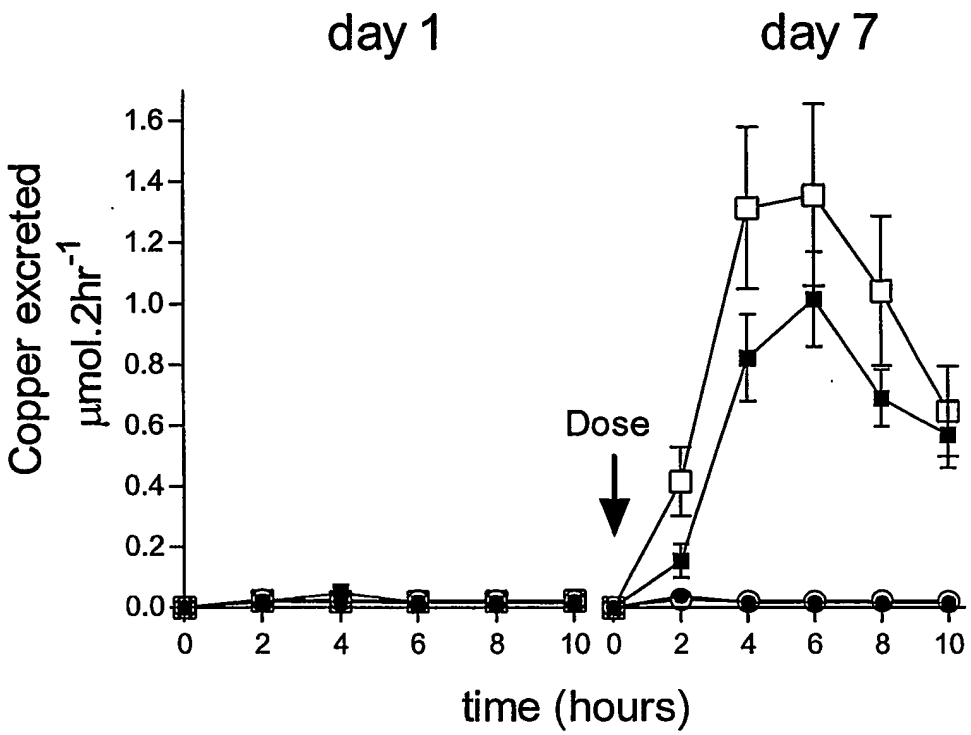

day 1
day 7
Copper excreted
µmol.2hr-1
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Dose
0
2
4
6
8
10
0
2
4
6
8
10
time (hours)

FIGURE 31

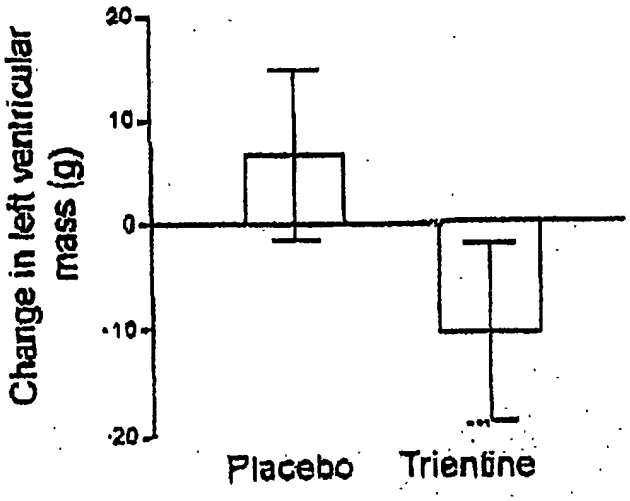

Change in left ventricular mass (g)
20
10
0
-10
-20
Placebo
Trientine

FIGURE 32

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ03/00047

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: A61K 31/198, 31/38, 33/30, 31/45, A61P 3/10, 9/00.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT, MEDLINE; keywords: myocard+, cardiomyopathy +, heart+, hyperten+, ater+, copper, Cu.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 6147070 A (Francesco Facchini) 14 November 2000. See whole document.	1-32
A	Berenshtein et al, 'Roles of ferritin and iron in ischemic preconditioning of the heart'. Molecular and cellular biochemistry 234/235, pp283-292, 2002. See whole document.	1-32

☐ Further documents are listed in the continuation of Box C ☒ See patent family annex

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 June 2003	16 JUN 2003
Name and mailing address of the ISA/AU	Authorized officer
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	**G.R.PETERS** Telephone No : (02) 6283 2184

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/NZ03/00047

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Member
US 6147070	AU 44215/99 WO 9962336

END OF ANNEX

increasing doses of trientine (0.1, 1.0, 10, 100 mg.kg^{-1} in 75 µl saline followed by 125 µl saline flush injected at time shown by arrow) or an equivalent volume of saline, and each point represents a 15 min urine collection period (see Methods for details); error bars show SEM and *P* values are stated if significant ($P < 0.05$).

Figure 23 shows the urinary iron excretion per gram of bodyweight in diabetic and non diabetic animals receiving trientine or saline, wherein urinary iron excretion per gram of bodyweight in diabetic and nondiabetic animals in response to increasing doses of trientine (*bottom;* 0.1, 1.0, 10, 100 mg.kg^{-1} in 75 µl saline followed by 125 µl saline flush injected at time shown by arrow) or an equivalent volume of saline *(top)*, and each point represents a 15 min urine collection period (see Methods for details); error bars show SEM and *P* values are stated if significant ($P < 0.05$).

Figure 24 shows the total urinary iron excretion in non diabetic and diabetic animals administered saline or drug, wherein total urinary iron excretion (µmol) in nondiabetic animals administered saline (black bar, n = 7) or trientine (hatched bar, n = 7) and in diabetic animals administered saline (grey bar, n = 7) or trientine (white bar, n = 7); error bars show SEM and *P* values are stated if significant ($P < 0.05$).

Figure 25 shows the total urinary iron excretion per gram of bodyweight in animals receiving trientine or saline, wherein Total urinary iron excretion per gram of bodyweight (µmol.gBW^{-1}) in animals receiving trientine (nondiabetic: hatched bar, n = 7; diabetic: white bar, n = 7) or saline (nondiabetic: black bar, n = 7; diabetic: grey bar, n = 7); error bars show SEM and *P* values are stated if significant ($P \leq 0.05$).

Figure 26 is a table comparing the copper and iron excretion in the animals receiving trientine or saline, which is a statistical analysis using a mixed linear model.

Figure 27 shows urinary [Cu] by AAS (△) and EPR (▲) following sequential 10 $mg.kg^{-1}$ (A) and 100 $mg.kg^{-1}$ (B) trientine boluses, as in Figure 19; (*inset*) background-corrected EPR signal from 75-min urine indicating presence of Cu^{II}-trientine.

Figure 28 shows the structure of LV-myocardium from STZ-diabetic and matched non-diabetic control rats following 7-w oral trientine treatment, wherein cardiac sections were cut following functional studies. Each image is representative of 5 independent sections per heart x 3 hearts per treatment. **a** — **d**, Laser confocal images of 120-μM LV sections co-stained for actin (Phalloidin-488, orange) and immunostained for β_1-integrin (CY5-conjugated secondary antibody, purple) (scale-bar = 33 μm). **a**, Untreated-control; **b**, Untreated-diabetic; **c**, Trientine treated diabetic; **d**, Trientine-treated non-diabetic control. **e** — **h**, TEM images of corresponding 70-nM sections stained with uranyl acetate/lead citrate (scale-bar = 158 nm); **e**, Untreated-control; **f**, Untreated-diabetic; **g**, Trientine-treated diabetic; **h**, Trientine-treated non-diabetic control.

Figure 29 shows plasma concentration-time profiles of trientine after oral administration to four male patients.

Figure 30 shows plasma concentration-time profiles of trientine after oral administration to four female patients.

Figure 31 shows a randomised, double blind, placebo-controlled trial comparing effects of oral trientine and placebo on urinary Cu excretion from male

humans with uncomplicated T2DM and matched non-diabetic controls, wherein urinary Cu excretion (μmol.2 h^{-1} on day 1 (baseline) and day 7 following a single 2.4-g oral dose of trientine or matched placebo to subjects described in Table 9, placebo- treated T2DM, ○, placebo-treated control, ●, trientine-treated T2DM, □; trientine treated control,■. Cu excretion from T2DM following trientine-treatment was significantly greater than that from trientine-treated non-diabetic controls ($P < 0.05$).

Figure 32 shows effect of 6 months' oral trientine treatment on LV mass in humans with T2DM, wherein trientine (600mg twice-daily) or matched placebo were administered to subjects with diabetes (n = 15) or matched controls (n = 15) in a double-blind, parallel-group study, and wherein differences in LV mass (g; mean and 95% confidence interval)were determined by tagged-cardiac MRI. ($P<0.005$ for trientine-treated v placebo)

DETAILED DESCRIPTION OF THE INVENTION

The invention is related to and describes the methods relating to discoveries surrounding increased tissue copper and mechanisms leading to tissue damage, including nerve and vascular damage, for example, diabetic nerve and/or vascular damage. It is believed, without wishing to be bound by any particular mechanism or theory of operation or effectiveness, that tissue accumulation of trace metals plays a role in the mechanisms of tissue damage in diabetes as well as in other disorders, diseases, and conditions as set forth or referenced or suggested herein.

Histological evidence from experiments showed that six months of treatment with trientine appears to protect the hearts of diabetic Wistar rats from development of diabetic damage (cardiomyopathy) as judged by histology. The doses of trientine required for copper and iron to be excreted in the urine have also been

investigated, for example, as well as possible differences between the excretion of these metals in diabetic and nondiabetic animals. For example, the excretion profiles of copper and iron in the urine of normal and diabetic rats were compared after acute intravenous administration of increasing doses of trientine. Additionally, it was ascertained whether acute intravenous administration of trientine has acute adverse cardiovascular side effects. Methods used in the experimentals were as follows.

Male Wistar rats (n = 28, 303 ± 2.9 g) were divided randomly into diabetic and nondiabetic groups. Following induction of anesthesia (5% halothane and 2l.min^{-1} O_2), animals in the diabetic group received a single intravenous dose of streptozotocin (STZ, 55mg.kg^{-1} body weight, Sigma; St. Louis, MO) in 0.5 ml saline administered via the tail vein. Nondiabetic animals received an equivalent volume of saline. Following injection, both diabetic and nondiabetic rats were housed in like-pairs and provided with access to normal rat chow (Diet 86 pellets; New Zealand Stock Feeds, Auckland, NZ) and deionized water *ad libitum*. Blood glucose and body weight were measure at day 3 following STZ/saline injection and then weekly throughout the study. Diabetes was identified by polydipsia, polyuria and hyperglycemia (> 11 mmol.l^{-1}, Advantage II, Roche Diagnostics, NZ Ltd).

Six to seven weeks (mean = 44 ± 1 days) after administration of STZ, animals underwent either a control or drug experimental protocol. All animals were fasted overnight prior to surgery but continued to have *ad libitum* access to deionized water. Induction and maintenance of surgical anesthesia was by 3 - 5% halothane and 2l.min^{-1} O_2. The femoral artery and vein were cannulated with a solid-state blood pressure transducer (Mikrotip™ 1.4F, Millar Instruments, Texas, USA) and a saline

filled PE 50 catheter respectively. The ureters were exposed via a midline abdominal incision, cannulated using polyethylene catheters (external diameter 0.9mm, internal diameter 0.5mm) and the wound sutured closed. The trachea was cannulated and the animal ventilated at 70-80 breaths.min^{-1} with air supplemented with O_2 (Pressure Controlled Ventilator, Kent Scientific, Connecticut, USA). The respiratory rate and end-tidal pressure (10-15 cmH_2O) were adjusted to maintain end-tidal CO_2 at 35-40 mm Hg (SC-300 CO_2 Monitor, Pryon Corporation, Wisconsin, USA). Body temperature was maintained at 37°C throughout surgery and the experiment by a heating pad. Estimated fluid loss was replaced with intravenous administration of 154 mmol.l^{-1} NaCl solution at a rate of 5 ml.kg^{-1}.h^{-1}.

Following surgery and a 20 min stabilization period, the experimental protocol was started. Trientine was administered intravenously over 60 s in hourly doses of increasing concentration (0.1, 1.0, 10 and 100 mg.kg^{-1} in 75 µl saline followed by 125 µl saline flush). Control animals received an equivalent volume of saline. Urine was collected in 15 min aliquots throughout the experiment in pre-weighed polyethylene epindorph tubes. At the end of the experiment a terminal blood sample was taken by cardiac puncture and the separated serum stored at -80°C until future analysis. Hearts were removed through a rapid mid-sternal thoracotomy and processed as described below.

Mean arterial pressure (MAP), heart rate (HR, derived from the MAP waveform) oxygen saturation (Nonin 8600V Pulse Oximeter, Nonin Medical Inc., Minnesota, USA) and core body temperature, were all continuously monitored throughout the experiment using a PowerLab/16s data acquisition module (AD

Instruments, Australia). Calibrated signals were displayed on screen and saved to disc as 2 s averages of each variable.

Instrumentation: A Perkin Elmer (PE) Model 3100 Atomic Absorption Spectrophotometer equipped with a PE HGA-600 Graphite Furnace and PE AS-60 Furnace Autosampler was used for Cu and Fe determinations in urine. Deuterium background correction was employed. A Cu or Fe hollow-cathode lamp (Perkin Elmer Corporation) was used and operated at either 10 W (Cu) or 15 W (Fe). The 324.8 nm atomic line was used for Cu and the 248.3 nm atomic line for Fe. The slit width for both Cu and Fe was 0.7 nm. Pyrolytically coated graphite tubes were used for all analyses. The injection volume was 20 µL. A typical graphite furnace temperature program is shown below.

GF-AAS temperature program

Procedure	*Temp / °C*	*Ramp / s*	*Hold / s*	*Int. Flow / mL min^{-1}*
Drying	90	1	5	300
	120	60	5	300
Pre-treatment	1250*	20	10	300
	20	1	10	300
Atomization – Cu / Fe	2300 / 2500	1	8	0
Post-treatment	2600	1	5	300

* A pre-treatment temperature of 1050 °C was used for tissue digest analyses

Cu, Fe and Zn in tissue digests were also determined at Hill Laboratories (Hamilton, New Zealand) using either a PE Sciex Elan-6000 or PE Sciex Elan-6100 DRC ICP-MS. The operating parameters are summarized in the table below.

Instrumental operating parameters for ICP-MS

Parameter	*Value*
Inductively coupled plasma	
Radiofrequency power	1500 W
Argon plasma gas flow rate	15 $l.min^{-1}$
Argon auxiliary gas flow rate	1.2 $l.min^{-1}$
Argon nebuliser gas flow rate	0.89 $l.min^{-1}$
Interface	
Sampler cone and orifice diameter	Ni / 1.1 mm
Skimmer cone and orifice diameter	Ni / 0.9 mm
Data acquisition parameters	
Scanning mode	Peak hopping
Dwell time	30 ms (Cu, Zn) / 100 ms (Fe)
Sweeps / replicate	20
Replicates	3
Sample uptake rate	1 $ml.min^{-1}$

Reagents: All reagents used were of the highest purity available and at least of analytical grade. GF-AAS standard working solutions of Cu and Fe were prepared by stepwise dilution of 1000 $mg.l^{-1}$ (Spectrosol standard solutions; BDH). Water was purified by a Millipore Milli-Q ultra-pure water system to a resistivity of 18 MΩ. Standard Reference Material 1577b Bovine Liver was obtained from the National Institute of Standards and Technology and used to evaluate the efficiency of tissue digestion. The results obtained are reported below.

GF-AAS and ICP-MS results for NIST SRM 1577b bovine liver*

Element	*Certified value*	*GF-AAS*	*ICP-MS*
Cu	160 ± 8	142 ± 12	164 ± 12
Fe	184 ± 15	182 ± 21	166 ± 14
Zn	127 ± 16	–	155 ± 42

* Measured in $\mu g.g^{-1}$ of dry matter.

Samples were pretreated as follows:

Urine: Urine was collected in pre-weighed 1.5 ml micro test tubes (eppendorf). After reweighing, the urine specimens were centrifuged and the supernatant diluted 25:1 with 0.02 M 69 % Aristar grade HNO_3. The sample was stored at 4 °C prior to GF-AAS analysis. If it was necessary to store a sample for a period in excess of 2 weeks, it was frozen and kept at –20 °C.

Heart: Following removal from the animal, the heart was cleaned of excess tissue, rinsed in buffer to remove excess blood, blotted dry and a wet ventricular weight recorded. Using titanium instruments a segment of left ventricular muscle was dissected and placed in a pre-weighed 5.0 ml polystyrene tube. The sample was freeze-dried overnight to constant weight before 0.45 ml of 69% Aristar grade HNO_3 was added. The sample tube was heated in a water bath at 65 °C for 60 minutes. The sample was brought to 4.5 ml with Milli-Q H_2O. The resulting solution was diluted 2:1 in order to reduce the HNO_3 concentration below the maximum permitted for ICP-MS analysis.

Serum: Terminal blood samples were centrifuged and serum treated and stored as per urine until analysis. From the trace metal content of serum from the

terminal blood sample and urine collected over the final hour of the experiment, renal clearance was calculated using the following equation: renal clearance of trace metal ($\mu l.min^{-1}$) = (a) the concentration of metal in urine ($\mu g.\ \mu l^{-1}$) times (b) the rate of urine flow ($\mu l.min^{-1}$), divided by (c) the concentration of metal in serum ($\mu g.\ \mu l^{-1}$)

Statistical analyses were as follows: All values are expressed as mean ± SEM and P values < 0.05 were considered statistically significant. Student's unpaired t-test was initially used to test for weight and glucose differences between the diabetic and control groups. For comparison of responses during drug exposure, statistical analyses were performed using analysis of variance (Statistica for Windows v.6.1, SAS Institute Inc., Calfornia, USA). Subsequent statistical analysis was performed using a mixed model repeated measures ANOVA design.

Statistical analysis using a mixed linear model: Data for each dose level were analyzed using a mixed linear model (PROC MIXED; SAS, Version 8). The model included *diabetes*, *drug* and their interaction as fixed effects, *time* as a repeated measure, and *rats* as the subjects in the dataset. Complete independence is assumed across subjects. The full model was fitted to each dataset using a maximum likelihood estimation method (REML) fits mixed linear models (i.e., fixed and random effects models). A mixed model is a generalization of the standard linear model, the generalization being that you can analyse data generated from several sources of variation instead of just one. A level of significance of 0.05 was used for all tests.

The results were as follows.

Effects of STZ on blood glucose and body weight (Table 1): Blood glucose increased to 25 ± 2 $mmol.l^{-1}$ three days following STZ injection. Despite a

Table 1. **Blood glucose, body weight and food consumption in diabetic versus nondiabetic animals.**

Diabetic animals n = 14, nondiabetic animals n = 14. Values shown as mean ± SEM. Asterisk indicates a significant difference ($P < 0.05$).

	I. DIABETIC	II. NONDIABETIC
Body weight prior to STZ/saline	303 ± 3 g	303 ± 3 g
Blood glucose 3 days following STZ/saline	*25 ± 2 mmol.l^{-1}	5 ± 0.2 mmol.l^{-1}
Daily food consumption	*58 ± 1 g	28 ± 1 g
Blood glucose on experimental day	*24 ± 1 mmol.l^{-1}	5 ± 0.2 mmol.l^{-1}
Body weight on experimental day	*264 ± 7 g	434 ± 9 g

greater daily food intake, diabetic animals lost weight whilst nondiabetic animals continued to gain weight during the 44 days following STZ/saline injection. On the day of the experiment blood glucose levels were 24 ± 1 and 5 ± 0 mmol.l^{-1} and body weight 264 ± 7 g and 434 ± 9 g for diabetic and nondiabetic animals respectively.

Cardiovascular variables during infusion: Baseline levels of MAP during the control period prior to infusion were not significantly different between nondiabetic and diabetic animals (99 ± 4 mm Hg). HR was significantly lower in diabetic than nondiabetic animals (287 ± 11 and 364 ± 9 bpm respectively, $P < 0.001$). Infusion of trientine or saline had no effect on these variables except at the highest dose where MAP decreased by a maximum of 19 ± 4 mm Hg for the 2 min following

administration and returned to pre-dose levels within 10 min. Body temperature and oxygen saturation remained stable in all animals throughout the experiment.

Urine excretion: Diabetic animals consistently excreted significantly more urine than nondiabetic animals except in response to the highest dose of drug (100 $mg.kg^{-1}$) or equivalent volume of saline (Fig. 16). Administration of the 100 $mg.kg^{-1}$ dose of trientine also increased urine excretion in nondiabetic animals to greater than that of nondiabetic animals receiving the equivalent volume of saline (Fig. 17). This effect was not seen in diabetic animals.

Urinary excretion of Cu and Fe: Analysis of the dose response curves shows that, at all doses, diabetic and nondiabetic animals receiving drug excreted more Cu than animals receiving an equivalent volume of saline (Fig. 18). To provide some correction for the effects of lesser total body growth of the diabetic animals, and thus to allow more appropriate comparison between diabetic and nondiabetic animals, excretion rates of trace elements were also calculated per gram of body weight. Figure 19 shows that diabetic animals had significantly greater copper excretion per gram of body weight in response to each dose of drug than did nondiabetic animals. The same pattern was seen in response to saline, however the effect was not always significant. Total copper excreted over the entire duration of the experiment was significantly increased in both nondiabetic and diabetic animals administered trientine compared with their respective saline controls (Fig. 20). Diabetic animals receiving drug also excreted more total copper per gram of body weight than nondiabetic animals receiving drug. The same significant trend was seen in response to saline administration (Fig. 21).

In comparison, iron excretion in both diabetic and nondiabetic animals receiving trientine was not greater than animals receiving an equivalent volume of saline (Fig. 22). Analysis per gram of body weight shows diabetic animals receiving saline excrete significantly more iron than nondiabetic animals, however this trend was not evident between diabetic and nondiabetic animals receiving trientine (Fig. 23). Total iron excretion in both diabetic and nondiabetic animals receiving drug was not different from animals receiving saline (Fig 24). In agreement with analysis of dose response curves, total iron excretion per gram of body weight was significantly greater

in diabetic animals receiving saline than nondiabetic animals but this difference was not seen in response to trientine (Fig. 25).

Electron paramagnetic resonance spectroscopy showed that the urinary Cu from drug-treated animals was mainly complexed as trientine-Cu^{II} (Fig. 27), indicating that the increased tissue Cu in diabetic rats is mainly divalent. These data indicate that rats with severe hyperglycaemia develop increased systemic Cu^{II} that can be extracted by selective chelation.

Serum content and renal clearance of Cu and Fe (Table 2): While there was no significant difference in serum copper content, there was a significant increase in renal clearance of copper in diabetic animals receiving drug compared with diabetic animals receiving saline. The same pattern was seen in nondiabetic animals, although the trend was not statistically significant ($P = 0.056$). There was no effect of drug or state (diabetic versus nondiabetic) on serum content or renal clearance of iron.

Table 2. **Serum content and renal clearance of Cu and Fe in diabetic and nondiabetic animals receiving drug or saline.**

	1.1.a.a.1 diabetic	*1.1.a.a.1 diabetic*	*1.1.a.a.2 nondiabetic*	*1.1.a.a.2 nondiabetic*
	trientine n = 6	*Saline n = 7*	*trientine n = 4*	*Saline n = 7*
Serum Cu ($\mu g.\mu l^{-1} \times 10^{-4}$)	7.56 ± 0.06	9.07 ± 1.74	7.11 ± 0.41	7.56 ± 0.62
Serum Fe ($\mu g.\mu l^{-1} \times 10^{-4}$)	35.7 ± 7.98	63.2 ± 16.4	33.6 ± 1.62	31.4 ± 8.17
Renal clearance Cu ($\mu l.min^{-1}$)	*28.5 ±	1.66 ±	19.9 ± 6.4	0.58 ± 0.28

	4.8	0.82		
Renal clearance Fe ($\mu l.min^{-1}$)	0.25 ± 0.07	0.38 ± 0.15	0.46 ± 0.22	0.11 ± 0.03

Values shown as mean ± SEM. Asterisk indicates a significant difference ($P < 0.05$) between diabetic animals receiving trientine and diabetic animals receiving an equivalent volume of saline.

Metal content of cardiac tissue (Table 3): Wet heart weights in diabetic animals were significantly less than those in nondiabetic animals while heart/body weight ratios were increased. Cardiac tissue was also analyzed for Cu and Fe content. There was no significant difference in content of copper between diabetic and nondiabetic animals receiving saline or trientine. Iron content of the non-diabetic animals administered saline was significantly greater than that of the diabetic animals administered saline.

Table 3. **Heart weight, heart weight/body weight ratios and trace metal content of heart tissue in diabetic versus nondiabetic animals.**

	DIABETIC	**NONDIABETIC**
Wet heart weight	*0.78 ± 0.02 g	1.00 ± 0.02 g
Heart weight/body weight	*2.93 ± 0.05 $mg.g^{-1}$	2.30 ± 0.03 $mg.g^{-1}$
Cu content $\mu g.g^{-1}$ dry tissue		
Trientine treated	24.7 ± 1.5	27.1± 1.0
Saline treated	21.3 ± 0.9	27.2 ± 0.7
Fe content $\mu g.g^{-1}$ dry tissue		
Trientine treated	186 ± 46	235 ± 39
Saline treated	†180 ± 35	274 ± 30

Diabetic animals: n = 14; nondiabetic animals: n = 14. Values shown as mean ± SEM. Asterisk indicates a significant difference ($P < 0.05$) between diabetic and non-diabetic animals.† indicates a significant difference ($P < 0.05$) between diabetic and non-diabetic animals receiving saline.

Results from application of a mixed linear model to the experimental analysis (Figure 26).

Copper: Diabetic rats excreted significantly higher levels of copper across all dose levels. Baseline copper excretion was also significantly higher in diabetic rats compared to non diabetic rats and prior to drug administration. The drug resulted in a significantly higher excretion of copper compared to saline at all dose levels. There was no difference at baseline levels between the drug and saline groups. The interaction effect for the model was significant at dose levels of 1.0 $mg.kg^{-1}$ and above. The presence of a significant interaction term means that the influence of one effect varies with the level of the other effect. Therefore, the outcome of a significant interaction between the diabetes and drug factors is increased copper excretion above the predicted additive effects of these two factors.

Iron: Diabetic rats in the saline only group excreted significantly higher levels of iron at all dose levels. This resulted in all factors in the model being significant across all dose levels.

In sum, the acute effect of intravenous trientine administration on the cardiovascular system and urinary excretion of copper and iron was studied in anesthetized, diabetic (6 weeks of diabetes, Streptozotocin induced) and nondiabetic rats. Animals were assigned to one of four groups: diabetic + trientine, diabetic + saline, nondiabetic + trientine, nondiabetic + saline. Drug, or an equivalent volume of saline, was administered hourly in doses of increasing strength (0.1, 1.0, 10, 100 $mg.kg^{-1}$) and urine was collected throughout the experiment in 15 min aliquots. A terminal

blood sample was taken and cardiac tissue harvested. Analysis of urine samples showed the following main points:

- At all drug doses, diabetic and nondiabetic animals receiving drug excreted more Cu (μmol) than animals receiving an equivalent volume of saline.
- When analyzed per gram of bodyweight, diabetic animals excreted significantly more copper ($\mu mol.gBW^{-1}$) at each dose of trientine than did nondiabetic animals. The same pattern was seen in response to saline but the effect was not significant at every dose.
- At most doses, in diabetic animals iron excretion (μmol) was greater in animals administered saline than in those administered drug. In nondiabetic animals there was no difference between iron excretion in response to saline or trientine administration.
- Analysis per gram of body weight shows no difference between iron excretion in nondiabetic and diabetic animals receiving trientine. Diabetic animals receiving saline excrete more iron per gram of bodyweight than nondiabetic animals receiving saline.
- Analysis of heart tissue showed no significant difference in total copper content between diabetic and nondiabetic animals, nor any effect of drug on cardiac content of iron and copper.
- Renal clearance calculations showed a significant increase in clearance of copper in diabetic animals receiving trientine compared with diabetic animals receiving saline. The same trend was seen in nondiabetic

animals but the affect was not significant. There was no effect of trientine on renal clearance of iron.

Thus, there were no adverse cardiovascular effects were observed after acute administration of trientine. Trientine treatment effectively increases copper excretion in both diabetic and nondiabetic animals. The excretion of copper in urine following trientine administration is greater per gram of bodyweight in diabetic than in nondiabetic animals. Iron excretion was not increased by trientine treatment in either diabetic or nondiabetic animals.

Experiments relating to the efficacy of trientine to restore cardiac function in STZ diabetic rats were also carried out. As noted above, histological evidence from earlier studies showed that treatment with trientine appears to protect the hearts of diabetic Wistar rats from development of cardiac damage (diabetic cardiomyopathy), as judged by histology. However, it was unknown whether this histological improvement translates into an improvement in cardiac function. One aim of this study was to use an isolated-working- rodent heart model to compare cardiac function in trientine-treated and non-treated, STZ diabetic and normal rats.

Male albino Wistar rats weighing 330-430g were assigned to four experimental groups as follows:

Experimental groups

Group	Code	N	Treatment
Group A	STZ	8	Diabetes for 13 weeks
Group B	STZ/D7	8	Diabetes for 13 weeks (Drug therapy week 7-13)
Group C	Sham	9	Non-diabetic controls

Group D	Sham/D7	11	Non-diabetic controls (Drug therapy week 7-13)

STZ = Streptozotocin; D7 = trientine treatment for 7 consecutive weeks commencing 6 weeks after the start of the experiment.

Diabetes was induced by intravenous streptozotocin (STZ; Sigma; St. Louis, MO). All rats were given a short inhalational anaesthetic (Induction: 5% halothane and 2L/min oxygen, maintained on 2% halothane and 2 L/min oxygen). Those in the two diabetic groups then received a single intravenous bolus dose of STZ (55mg/kg body weight) in 0.5 ml of 0.9% saline administered via a tail vein. Non-diabetic sham-treated animals received an equivalent volume of 0.9% saline. Diabetic and non-diabetic rats were housed in like-pairs and provided with free access to normal rat chow (Diet 86 pellets; New Zealand Stock Feeds, Auckland, NZ) and deionized water *ad libitum*. Each cage had two water bottles on it to ensure equal access to water or drug for each animal. Animals were housed at 21 degrees and 60% humidity in standard rat cages with a sawdust floor that was changed daily.

Blood glucose was measured in tail-tip capillary blood samples (Advantage II, Roche Diagnostics, NZ Ltd). Sampling was performed on all groups at the same time of the day. Blood glucose and body weight were measured on day 3 following STZ/saline injection and then weekly throughout the study. Diabetes was confirmed by presence of polydipsia, polyuria and hyperglycemia (>11mmol.L^{-1}).

In the drug treated diabetic group, trientine was prepared in the drinking water for each cage at a concentration of 50mg/L. Each animal consumed about 260ml water per day once diabetes was established, to yield a total drug dose per animal per day of ~13mg. The trientine-containing drinking water was administered continuously from the start of week 7 until the animal was sacrificed at the end of week

13. In the case of the Sham/D7 non-diabetic group that drank less water per day than diabetic animals, the drug concentration in their drinking water was adjusted so that they consumed approximately the same dose as the corresponding STZ/D7 group. "Trientine treated animals ingested mean drug doses of between 8 to 11mg per day."

At the time the drug started in the diabetic group the diabetic animals were expected to have to have established cardiomyopathy, as shown by preliminary studies (data not shown) and confirmed in the literature. See Rodrigues B, *et al.*, *Diabetes* 37(10):1358-64 (1988).

On the last day of the experiment, animals were anesthetized (5% halothane and 2L.min^{-1} O_2), and heparin (500 IU.kg^{-1}) (Weddel Pharmaceutical Ltd., London) administered intravenously via tail vein. A 2ml blood sample was then taken from the inferior vena cava and the heart was then rapidly excised and immersed in ice-cold Krebs-Henseleit bicarbonate buffer to arrest contractile activity. Hearts were then placed in the isolated perfused working heart apparatus.

The aortic root of the heart was immediately ligated to the aortic cannula of the perfusion apparatus. Retrograde (Langendorff) perfusion at a hydrostatic pressure of 100 cm H_2O and at 37^{O}C was established and continued for 5min while cannulation of the left atrium via the pulmonary vein was completed. The non-working (Langendorff) preparation was then converted to the working heart model by switching the supply of perfusate buffer from the aorta to the left atrium at a filling pressure of 10 cm H_2O. The left ventricle spontaneously ejected into the aortic cannula against a hydrostatic pressure (after-load) of 76 cmH_2O (55.9mmHg). The perfusion solution was Krebs-Henseleit bicarbonate buffer (mM: KCl 4.7, $CaCl_2$ 2.3, KH_2PO_4 1.2, $MgSO_4$ 1.2, NaCl 118, and $NaHCO_3$ 25), pH 7.4 containing 11mM glucose and it was

continuously gassed with 95% O_2:5% CO_2. The buffer was also continuously filtered in-line (initial 8μm, following 0.4μm cellulose acetate filters; Sartorius, Germany). The temperature of the entire perfusion apparatus was maintained by water jackets and buffer temperature was continuously monitored and adjusted to maintain hearts at 37°C throughout perfusion.

A modified 24g plastic intravenous cannula (Becton Dickson, Utah, USA) was inserted into the left ventricle via the apex of the heart using the normal introducer-needle. This cannula was subsequently attached to a SP844 piezo-electric pressure transducer (AD Instruments) to continuously monitor left ventricular pressure. Aortic pressure was continuously monitored through a side arm of the aortic cannula with a pressure transducer (Statham Model P23XL, Gould Inc., CA, USA). The heart was paced (Digitimer Ltd, Heredfordshire, England) at a rate of 300bpm by means of electrodes attached to the aortic and pulmonary vein cannulae using supra-threshold voltages with pulses of 5-ms duration from the square wave generator.

Aortic flow was recorded by an in-line flow meter (Transonic T206, Ithaca, NY, USA) and coronary flow was measured by timed 30sec collection of the coronary vein effluent at each time point step of the protocol.

The working heart apparatus used was a variant of that originally described by Neely, JR, *et al.*, *Am J Physiol* **212**:804-14 (1967). The modified apparatus allowed measurements of cardiac function at different pre-load pressures (Figure 14 and Figure 15). This was achieved by constructing the apparatus so that the inflow height of the buffer coming to the heart could be altered through a series of graduated steps in a reproducible manner. As in the case of the pre-load, the outflow

tubing from the aorta could also be increased in height to provide a series of defined after-load pressures. The after-load heights have been converted to mm Hg for presentation in the results which is in keeping with published convention.

All data from the pressure transducers and flow probe were collected (Powerlab 16s data acquisition machine; AD Instruments, Australia). The data processing functions of this device were used to calculate the first derivative of the two pressure waves (ventricular and aortic). The final cardiac function data available comprised:

Cardiac output*; aortic flow; coronary flow; peak left ventricular/aortic pressure developed; maximum rate of ventricular pressure development (+dP/dt)**; maximum rate of ventricular pressure relaxation (-dP/dt)**; maximum rate of aortic pressure development (aortic +dP/dt); maximum rate of aortic relaxation (aortic -dP/dt).

[*Cardiac output (CO) is the amount of buffer pumped per unit time by the heart and is comprised of buffer that is pumped out the aorta as well as the buffer pumped into the coronary vessels. This is an overall indicator of cardiac function. ** +dP/dt is the rate of change of ventricular (or aortic pressure) and correlates well with the strength of the contraction of the ventricle (contractility). It can be used to compare contractility abilities of different hearts when at the same pre-load (Textbook of Medical Physiology, Ed. A.Guyton. Saunders company 1986). -dP/dt is an accepted measurement of the rate of relaxation of the ventricle].

The experiment was divided into two parts, the first with fixed after-load and variable pre-load the second, which immediately followed on from the first, with fixed pre-load and variable after-load.

Fixed After-load and changing Pre-load: After the initial cannulation was completed, the heart was initially allowed to equilibrate for 6min at 10cm H_2O atrial filling pressure and 76cm H_2O after-load. During this period the left ventricular pressure transducer cannula was inserted and the pacing unit started. Once the heart was stable, the atrial filling pressure was then reduced to 5cm H_2O of water and then progressively increased in steps of 2.5cmH_2O over a series of 7 steps to a maximum of 20cmH_2O. The pre-load was kept at each filling pressure for 2min, during which time the pressure trace could be observed to stabilize and the coronary flow was measured. On completion of the variable pre-load experiment, the variable after-load portion of the experiment was immediately commenced.

Fixed Pre-load and changing After-load: During this part of the experiment the filling pressure (pre-load) was set at 10cm H_2O and the after-load was then increased from 76cm H_2O (55.9 mm Hg) in steps of 8cm H_2O (5.88mmHg); again each step was of 2min duration. The maximum height (after-load) to which each individual heart was ultimately exposed, was determined either by attainment of the maximal available after-load height of 145cm H_2O (106.66 mm Hg), or the height at which measured aortic flow became 0 ml/min. In the later situation, the heart was considered to have "functionally failed." To ensure that this failure was indeed functional and not due to other causes (*e.g.*, permanent ischaemic or valvular damage) all hearts were then returned to the initial perfusion conditions (pre-load 10cm H2O;

after-load 75 cm H2O) for 4 minutes to confirm that pump function could be restored. At the end of this period the hearts were arrested with a retrograde infusion of 4ml of cold KCL (24mM). The atria and vascular remnants were then excised, the heart blotted dry and weighed. The ventricles were incised midway between the apex and atrioventricular sulcus. Measurements of the ventricular wall thickness were then made using a micro-caliper (Absolute Digimatic, Mitutoyo Corp, Japan).

Data from the Powerlab was extracted by averaging 1min intervals from the stable part of the electronic trace generated from each step in the protocol. The results from each group were then combined and analyzed for differences between the groups for the various cardiac function parameters (aortic flow, cardiac flow, MLVDP, LV or aortic +/-dP/dt). Differences between repeated observations at different pre-load conditions were explored and contrasted between study group using a mixed models approach to repeated measures (SAS v8.1, SAS Institute Inc, Cary NC). Missing random data were imputed using a maximum likelihood approach. Significant mean and interaction effects were further examined using the method of Tukey to maintain a pairwise 5% error rate for post hoc tests. All tests were two-tailed. Survival analysis was done using Proc Liftest (SAS V8.2). A one-way analysis of variance was used to test for difference between groups in various weight parameters. Tukey's tests were used to compare each group with each other. In each graph unless otherwise stated.* indicates $p<0.05$ = STZ v STZ/D7, #.$p<0.05$ = STZ/D7 v Sham/D7.

Results showing that the weights of the animals at the end of the experimental period are found in Table 4. Diabetic animals were about 50% smaller than their corresponding age matched normals. A graph of the percentage change in

weight for each experimental group is found in Figure 5, wherein the arrow indicates the start of trientine treatment.

Table 4. Initial and final animal body weights (mean ± SD)

	Number (n)	Treatment	Initial weight (g)	Final weight (g)
Group A	8	STZ	361 ±12	221±27
* Group B	8	STZ/D7	401 ± 33	290±56
* Group C	9	Sham	361 ±16	574±50
Group D	11	Sham/D7	357 ±7	563±17

*P < 0.05

Blood glucose values for the three groups of rats are presented in Figure 6. Generally, the presence of diabetes was established and confirmed within 3-5 days following the STZ injection. The Sham and Sham/D7 control group remained normoglycemic throughout the experiment. Treatment with the drug made no difference to the blood glucose profile (p=ns) in either treated group compared to their respective appropriate untreated comparison group.

Final heart weight and ventricular wall thickness measurements are presented in Table 5. There was a small but significant improvement in the "heart : body weight" ratio with treatment in the diabetic animals. There was a trend toward improved "ventricular wall thickness:bodyweight" ratio in treated diabetics compared to non-treated but this did not reach significance.

Table 5 Final heart weights (g) and per g of animal body Weight (BW) (mean ± SD) ns

Group	Heart weight (g)	Heart weight (g) /BW (g)	Left Ventricular wall thickness (mm)	Left Ventricular wall thickness per BW (mm)/ (g)

			(mm)	
Sham	1.58 ± 0.13§	0.0028±0.0002§	3.89±0.38§	0.0068±0.0009§
STZ/D7	1.18 ± .24	0.0041±0.0005	3.79±0.52	0.0127±0.0027
	ns	*	ns	ns
STZ	1.03 ± 0.17	0.0047±0.0004	3.31±0.39	0.0152±0.0026
Sham/D7	1.58 ± 0.05§	0.0028±0.0001§	4.03±0.1§	0.0072±0.0003§

* P<0.05

§ = significant with the STZ and STZ/D7 groups p<0.05

Part I results: The following graphs of Figures 7 to 12 represent cardiac performance parameters of the animals (STZ diabetic; STZ diabetic +drug; and sham-treated controls) while undergoing increasing atrial filling pressure (5-20 cmH_2O, pre-load) with a constant after-load of 75cm H_2O. All results are mean ± sem. In each graph for clarity unless otherwise stated, only significant differences related to the STZ/D7 the other groups are shown:* indicates p<0.05 for STZ v STZ/D7, # p<0.05 for STZ/D7 v Sham/D7. Unless stated, STZ/D7 v Sham or Sham/D7 was not significant.

Cardiac output (Figure 7) is the sum to the aortic flow (Figure 10) and the coronary flow as displayed in Figure 8. Since the control hearts and experimental groups have significantly different final weights, the coronary flow is also presented (Figure 9) as the flow normalized to heart weight (note that coronary flow is generally proportional to cardiac muscle mass, and therefore to cardiac weight)

The first derivative of the pressure curve gives the rate of change in pressure development in the ventricle with each cardiac cycle and the maximum positive rate of change (+dP/dt) value is plotted in Figure 11. The corresponding

maximum rate of relaxation (-dP/dt) is in Figure 12. Similar results showing improvement in cardiac function were found from the data derived from the aortic pressure cannula (results not shown).

Part II results:

Under conditions for constant pre-load and increasing after-load the ability of the hearts to cope with additional after-load work was assessed. The plot of functional survival, that is the remaining number of hearts at each after-load that still had an aortic output of greater than 0ml/min is found in Figure 13

a.	Number surviving (aortic flow >0mls/min)				b.	Percentage functioning at each afterload		
Afterload (mmHg)	**STZ**	**STZ/D7**	**Sham**	**Sham/D7**	**STZ**	**STZ/D7**	**Sham**	**Sham/D7**
55.9	8	8	9	11	100%	100%	100%	100%
61.8	8	8	9	11	100%	100%	100%	100%
67.7	8	8	9	11	100%	100%	100%	100%
71.4	6	8	9	11	75%	100%	100%	100%
77.2	5	8	9	11	63%	100%	100%	100%
83.1	4	7	9	11	50%	88%	100%	100%
88.3	3	7	9	11	38%	88%	100%	100%
94.9	1	5	9	11	13%	62.575%	100%	100%
100.8	0	3	9	11	0%	37%	100%	100%
106.7	0	1	9	9	0%	13%	100%	82%

Cu chelation normalizes LV structure in diabetic rats

Following functional analysis, LV histology was studied by laser confocal (LCM; Fig. 28a - d) and transmission electron microscopy (TEM; Fig 28e - h). For

LCM, LV sections were co-stained with phalloidin to visualize actin filaments, and β_1-integrin as a marker for the extracellular space. Ding B, et al., "Left ventricular hypertrophy in ascending aortic stenosis in mice: anoikis and the progression to early failure," *Circulation* **101**:2854-2862 (2000).

For each treatment, 5 sections from each of 3 hearts were examined by both LCM and TEM. For LCM, LV sections were fixed (4% paraformaldehyde, 24 h); embedded (6% agar); vibratomed (120 pm, Campden); stained for f-actin (Phalloidin-488, Molecular Probes) and β_1-integrin antibody with a secondary antibody of goat anti-rabbit conjugated to CY5 (1:200; Ding B, et al., "Left ventricular hypertrophy in ascending aortic stenosis in mice: anoikis and the progression to early failure," *Circulation* **101**:2854-2862 (2000)); and visualised (TCS-SP2, Leica). For TEM, specimens were post-fixed (1:1 v/v 1% w/v 0s0 M 0s0 M PBS); stained (aqueous uranyl acetate (2 % w/v, 20 mm) then lead citrate (3 mm)); sectioned (70 nm); and visualized (CM-12, Phillips).

Compared with controls (Fig. 28a), diabetes caused obvious alterations in myocardial structure, with marked loss of myocytes; thinning and disorganization of remaining myofibrils; decreased density of actin filaments; and marked expansion of the interstitial space (Fig. 28b). These findings are consistent with previous reports. Jackson CV, et al., "A functional and ultrastructural analysis of experimental diabetic rat myocardium: manifestation of acardiomyopathy," *Diabetes* **34**:876-883 (1985). By marked contrast, myocardial histology following trientine treatment was essentially normal (Fig. 28c). Importantly, the orientation and volume of card iomyocytes and their actin filaments was largely normalized, consistent with the normalization of -dP_{LV}/dt observed in the functional studies. Trientine treatment reversed the expanded cardiac ECM. Myocardium from trientine-treated non-diabetics appeared normal by LCM (Fig. 28d) suggesting that it has no detectable adverse effects on LV structure. Thus, Cu chelation essentially restored the normal histological appearance of the myocardium without suppressing hyperglycaemia. These data provide important structural correlates for the functional recovery of these hearts, shown above.

TEM was largely consistent with LCM. Compared with controls (Fig. 28e), diabetes caused unmistakable myocardial damage characterized by loss of myocytes with evident myocytolysis; disorganization of remaining cardiomyocytes in which swollen mitochondria were prominent; and marked expansion of the extracellular space (Fig. 28f). These findings are consistent with previous reports. Jackson CV, et al., "A functional and ultrastructural analysis of experimental diabetic rat myocardium: manifestation of acardiomyopathy," *Diabetes* **34**:876-883 (1985). Oral trientine caused substantive recovery of LV structure in diabetics, with increased numbers and normalized orientation of myocytes; return to normal of mitochondrial structure; and marked narrowing of the extracellular space (Fig. 28g). These data suggest that hyperglycaemia-induced systemic Cu^{II} accumulation might contribute to the development of mitochondrial dysfunction. Brownlee M, "Biochemistry and molecular cell biology of diabetic complications," *Nature* **414**:813-820 (2001). Myocardium from trientine-treated non- diabetics appeared normal by TEM (Fig. 28h). Thus, trientine treatment normalized both cellular and interstitial aspects of hyperglycaemia-induced myocardial damage. Taken together, these microscopic studies provide remarkable evidence that selective Cu- chelation can normalize LV structure, even in the presence of severe chronic hyperglycaemia.

In sum, for example,

- Treatment with trientine had no obvious effect on blood glucose concentrations in the two diabetic groups (as expected).
- There was a small but significant improvement in the (heart weight) / (body weight) ratio in the trientine-treated diabetic group compared to that of the untreated diabetic group.
- When the Pre-load was increased with the After-load held constant, cardiac output was restored to Sham values. Both the aortic and absolute coronary flows improved in the drug treated group.

- Indicators for ventricular contraction and relaxation were both significantly improved in the drug treated group compared to equivalent values in the untreated diabetic group. The improvement restored function to such an extent that there was no significant difference between the drug treated and the sham-treated control groups.
- The aortic transducer measures of pressure change also showed improved function in the drug treated diabetic group compared to the untreated diabetics (data not shown).
- When after-load was increased in the presence of constant pre-load, it was observed that the heart's ability to function at higher after-loads was greatly improved in the drug treated diabetic group compared to the untreated diabetic group. When 50% of the untreated diabetic hearts had failed, about 90% of the trientine treated diabetic hearts were still functioning.
- Compared to the untreated diabetic hearts, the response of the drug treated diabetic hearts showed significant improvements in several variables: cardiac output, aortic flow, coronary flow, as well as improved ventricular contraction and relaxation indices.
- Drug treatment of normal animals had no adverse effects on cardiac performance.

It is concluded that treatment of STZ diabetic rats with trientine dramatically improves several measures of cardiac function. It is also concluded that administration of oral trientine for 7 weeks in Wistar rats with previously established diabetes of 6 weeks duration resulted in a global improvement in cardiac function. This

improvement was demonstrated by improved contractile function (; +dP/dT) and a reduction in ventricular stiffness (-dP/dT). The overall ability of the Trientine treated diabetic heart to tolerate increasing after-load was also substantially improved.

- **HUMAN STUDIES – Phase II**

Trace element balance in humans

Human studies were approved by institutional ethics and regulatory committees. Males with uncomplicated T2DM (Table 9) underwent 12-d elemental balance studies in a fully-residential metabolic unit. All foods and beverages were provided.

Total daily intake (method of double diets) and excretion (urinary and faecal) of trace elements (Ca, Mg, Zn, Fe, Cu, Mn, Mo, Cr and Se) were determined (ICP MS). Baseline measurements were taken during the first 6 d, after which oral trientine (2.4 g once-daily) or matched placebo was administered in a 2 x 2 randomised double-blind protocol and metal losses measured for a further 6 d.

Effects of chronic trientine treatment on LV mass in humans

Subjects (30-70 y) who provided written informed consent were eligible for inclusion if they had:

T2DM with HbA_{1c} >7%; cardiac ejection fraction (echocardiography) =45% with evidence of diastolic dysfunction but no regional wall-motion anomalies; no new medications for more than 6 months with no change of ß-blocker dose; normal electrocardiogram (sinus rhythm, normal PR Interval, normal T wave and QRS configuration, and isoelectric ST segment); and greater than 90% compliance with single-blinded placebo therapy during a 2-w run-in period). Women were required to be

post-menopausal, surgically sterile, or non-lactating and non-pregnant and using adequate contraception. Patients were ineligible if they failed to meet the inclusion criteria or had: morbid obesity (B. M. I. = 45 $kg.m^{-2}$ T1 DM; a history of significant cardiac valvular disease; evidence of autonomic neuropathy; ventricular wall motion abnormality; history of multiple drug allergies; use or misuse of substances of abuse; abnormal laboratory tests at randomisation; or standard contraindications to MRI.

Before randomisation, potentially eligible subjects entered a 4-w single blind run-in phase of two placebo-capsules twice-daily and underwent screening echocardiography, being excluded if regional wall motion abnormalities or impaired LV systolic function (ejection fraction <50%) were detected. In addition, LV diastolic filling was assessed using mitral inflow Doppler (with pre-load reduction) to ensure patients had abnormalities of diastolic filling; no patient with normal mitral filling proceeded to randomisation. Subjects meeting inclusion criteria and with no grounds for exclusion were then randomised to receive trientine (600 mg twice-daily) before meals (total dose 1.2 $g.d^{-1}$) or 2 identical placebo capsules twice-daily before meals, in a double-blind, parallel-group design. Treatment assignment was performed centrally using variable block sizes to ensure balance throughout trial recruitment and numbered drug packs were prepared and dispensed sequentially to randomised patients. The double-blind treatment was continued for 6 months in each subject.

At baseline and following 6 months' treatment, LV mass was determined using cardiac MRI, performed in the supine position with the same 1.5 T scanner (Siemens Vision) using a phased array surface coil. Prospectively gated cardiac cine images were acquired in 6 short axis and 3 long axis slices with the use of a segmented

k-space pulse sequence (TR 8 ms; TE 5 ms; flip angle 10°; field of view 280 - 350 mm) with view sharing (11 - 19 frames.slice^{-1}). Each slice was obtained during a breath-hold of 15 - 19 heart beats. The short axis slices spanned the left ventricle from apex to base with a slice thickness of 8 mm and inter-slice gap of 2 - 6 mm. The long axis slices were positioned at equal 60° intervals about the long axis of the LV. Cardiac MRI provides accurate and reproducible estimates of LV mass and volume. LV-mass and volume were calculated using guide point modelling, which produces precise and accurate estimations of mass and volume. Briefly, a three dimensional mathematical model of the LV was interactively fitted to the epicardial and endocardial boundaries of the LV wall in each slice of the study, simultaneously. Volume and mass were then calculated from the model by numerical integration (mass = wall volume x 1.05 g.ml^{-1}). All measurements were performed by 1 measurer at the end of six months' data collection. Outcome analyses were conducted by intention-to-treat, using a maximum likelihood approach to impute missing at random data within a mixed model, and marginal least-squares adjusted-means were determined. Changes from baseline were compared between treatment-groups in the mixed model with baseline values entered as covariate. Since there were only 2 groups in the main effect and no interaction effect, no *post hoc* procedures were employed. In additional analysis the influence of clinically important differences between the treatment groups at baseline was considered by adjusting for them as covariates in an additional model. All *P* values were calculated from 2-tailed tests of statistical significance and a 5% significance level was maintained throughout. The effect of treatment on categorical variables was tested using the procedures of Mantel and Haenzel (SAS v8.01, SAS Institute).

Table 7 shows baseline information on 30 patients with long-standing type 2 diabetes, no clinical evidence of coronary artery disease and abnormal diastolic function who participated in a 6-month randomised, double blind, placebo controlled study of chronic oral therapy with trientine dihydrochloride.

- **Table 7: Characteristics of Study Participants**

	Placebo	Trientine dihydrochloride
N	15	15
Median age (years)	54 (range 43-64)	52 (range 33-69)
% female	44%	56%
Median duration of diabetes (years)	10 (6-24)	8 (4-15)
Mean body mass index (kg/m^2)	32 (SD 5)	34 (SD 5)
% hypertensive	64%	80%
% HbA_{1c}>8	93%	80%

MRI scans of the heart at baseline and 6-months showed a significant reduction in LV mass and a significant improvement in diastolic function measured as a change in apical rotation (AR) at the end of systole. See, Table 8. These effects indicate improved structure and function in the human heart following 6 months of trientine therapy.

Table 8 : Phase II: INFO-Cardiac

MRI Results

	Placebo (n=15)	GC811007 (n=15)	P
Baseline LVM	202.17	207.45	0.778
Δ LVM 1-6mo	+6.57	-10.49	0.0045

Baseline AR	12.37	12.49	0.931
Δ AR 1-6mo	+0.81	-2.19	0.029

Therefore, an equivalent dose of oral trientine dihydrochloride corrected for weight (15 mg/kg) is effective in both rats and humans.

With reference to Figures 29 and 30 there is shown the plasma concentration – time profiles of trientine after oral administration. The plasma concentration was determined using the process as defined in Miyazaki K, et al., "Determination of trientine in plasma of patients with high-performance liquid chromatography," *Chem. Pharm. Bull.* **38**:1035-1038 (1990).

Urinary Cu losses are increased following oral trientine treatment in humans with type-2 diabetes

We measured urinary metal excretion in human males with T2DM or matched non-diabetic controls, baseline information on which is shown in Table 9, in a randomised, double blind, placebo-controlled trial.

- **Table 9: Characteristics of Study Participants**

	Placebo control	Trientine treated control	Placebo diabetic	Trientine treated diabetic
Median age (years)	42 (range 32 – 53)	52 (range 30 – 68)	51 (range 32 – 66)	50 (range 30 - 64)
n	10	10	10	10
Median duration of diabetes (years)	-	-	5.9 (range 1 - 13)	7.5 (range 1 - 34)
Fasting blood glucose ($mmol.L^{-1}$)	4.7 ± 0.3	5.0 ± 0.3	11.5 ± 3.8	10.8 ± 4.4
Mean HbA_{1c} (%)	5.4 ± 0.3	5.0 ± 0.3	9.9 ± 2.8	9.1 ± 1.6
Body mass index ($kg.m^{-2}$)	24.6 ± 3.5	27.9 ± 5.2	32.8 ± 4.4	30.4 ± 3.2

(mean ± S. E. M. unless otherwise stated); f. p. g., HbA_{1c} and B. M. I. were significantly greater in diabetics and groups were otherwise well-matched).

Basal 2-h Cu-losses were measured for 10 h in diabetic (n = 20) and matched control (n = 20) subjects during part of day I (Fig. 31); and daily losses were determined throughout days 1 - 6. Urine volumes were equivalent in drug- and placebo-treated groups. Baseline urinary Cu-excretion was significantly greater in diabetics than controls (mean diabetic, 0.257 $\mu mol.d^{-1}$ control, 0.196; $P < 0.001$). Trientine- and placebo-evoked 2-h urinary Cu-excretion was measured again in the same subjects on day 7 following oral drug (2.4 g once-daily) or matched placebo ($n = 10.group^{-1}$. Trientine increased urinary Cu in both groups, but the excretion rate in diabetes was greater (Fig 29; $P < 0.05$). There was no corresponding increase in trientine-evoked urinary Fe excretion, although basal concentrations in diabetes were increased relative to control ($P < 0.001$; results not shown). Thus, trientine elicited similar urinary Cu responses in rats with T1DM and in humans with T2DM. Mean trientine-evoked urinary Cu-excretion was 5.8 $\mu mol.d^{-1}$; in T2DM compared to 4.1 in non-diabetic controls, a 40 % increase. This correspondence between the two major forms of diabetes in two species suggests that increased systemic Cu^{II} is likely to be widely present in diabetes.

Trientine treatment reverses LVH in type-2 diabetic humans

We determined the effect of chronic trientine on LV mass in adults with T2DM, baseline information of which is shown in Table 10.

Table 10 Characteristics of Study Participants

	Placebo	Trientine-treated
Median age (years)	54 (range 43 – 64)	52 (range 33 - 69)
N	15	15

Female (%)	44	56
Median duration of diabetes (years)	10 (range 1- 24)	8 (range 1 -21)
Mean % HbA_{1c} (SD)	9.3 (1.3)	9.3 (2.0)
Initial left ventricular mass (g) (SD)	202.2 (53.1)	207.5 (48.7)
Δ urinary copper ($\mu mol.L^{-1}$)	0.67 (-1.16 to 2.49)	-0.83 (-2.4 to 0.74)
9 Δ systolic blood pressure (mmHg)	-1.9 (-10.6 to 6.8)	-3.5 (-9.5 to 1.8)
Δ diastolic blood pressure (mmHg)	-4.5 (-9.0 to 0.01)	-3.9 (-13.4 to 6.5)
Δ left ventricular mass/body surface area ($g.m^{-2}$)	+3.49 (0.63 to 7.61)	-5.56** (-9.64 to -1.48)

baseline values (*above line*); differences in key treatment-variables (6 months — baseline, mean (95% confidence interval; *below line*: **, $P < 0.01$ vs. placebo).

Trientine (600 mg twice-daily, a dose at the lower end of those employed in adult Wilson's disease, see Dahlman T, et al., "Long-term treatment of Wilson's disease with triethylene tetramine dihydrochloride (trientine)," *Quart. J. Med* **88**: 609-616 (1995)) or placebo were administered orally for 6 months to equivalent groups of diabetic adults ($n = 15.group^{-1}$; Table 10), also matched for pharmacotherapy including: β-blockers, calcium antagonists, ACE-inhibitors, cholesterol-lowering drugs, antiplatelet agents and antidiabetic drugs. LV masses were determined by tagged-molecular resonance imaging (MRI; see Bottini PB, et al., "Magnetic resonance imaging compared to echocardiography to assess left ventricular mass in the hypertensive patient," *Am. J. Hypertens* **8**: 221-228 (1995)) at baseline and following 6

months' trientine treatment. As expected, diabetics initially had significant LVH, consistent with previous reports. Struthers AD & Morris AD, "Screening for and treating left-ventricular abnormalities in diabetes mellitus: a new way of reducing cardiac deaths," *Lancet* **359**: 1430-1432 (2002). Mean LV mass significantly decreased, by 5%, following 6 months' trientine treatment, whereas that in placebo-treated subjects increased by 3% (Fig. 32); this highly significant effect remained after LV mass was indexed to body surface area, and occurred without change in systolic or diastolic blood pressure (Table 10). Thus, trientine caused powerful regression in LV mass without altering blood pressure or urinary volume (Fig. 31). No significant drug-related adverse events occurred during the 6 months' trientine therapy.

These data, taken together with those in rats above, indicate that chronic Cu chelation can cause significant regeneration of the heart in those with diabetes-evoked damage.

It is concluded that rats and humans with diabetes acquire increased systemic Cu^{II}, which can be removed by treatment with the Cu-selective chelator, trientine. Trientine largely reversed heart failure and LV damage in severely diabetic rats. Furthermore, 6 months' oral trientine therapy significantly ameliorated left ventricular hypertrophy in humans with type-2 diabetes.

Therapeutic formulations for use in the methods and preparation of the compositions of the present invention can be prepared by any methods well known in the art of pharmacy. See, for example, Gilman *et al.* (eds.) GOODMAN AND GILMAN'S: THE PHARMACOLOGICAL BASES OF THERAPEUTICS (8th ed.) Pergamon Press (1990); and Remington, THE SCIENCE OF PRACTICE AND PHARMACY, 20th Edition. (2001) Mack

Publishing Co., Easton, Pa.; Avis *et al.* (eds.) (1993) PHARMACEUTICAL DOSAGE FORMS: PARENTERAL MEDICATIONS Dekker, N.Y.; Lieberman *et al.* (eds.) (1990) PHARMACEUTICAL DOSAGE FORMS: TABLETS Dekker, N.Y.; and Lieberman *et al.* (eds.) (1990) PHARMACEUTICAL DOSAGE FORMS: DISPERSE SYSTEMS Dekker, N.Y. Dosage forms useful herein include any appropriate dosage form well known in the art to be suitable for pharmaceutical formulation of compounds suitable for administration to mammals particularly humans, particularly (although not solely) those suitable for stabilization in solution of therapeutic compounds for administration to mammals preferably humans. The dosage forms of the invention thus include any appropriate dosage form now known or later discovered in the art to be suitable for pharmaceutical formulation of compounds suitable for administration to mammals particularly humans, particularly (although not solely) those suitable for stabilization in solution of compounds for administration to mammals preferably humans. One example is oral delivery forms of tablet, capsule, lozenge, or the like form, or any liquid form such as syrups, aqueous solutions, emulsion and the like, capable of protecting the compound from degradation prior to eliciting an effect, for example, in the alimentary canal if an oral dosage form. Examples of dosage forms for transdermal delivery include transdermal patches, transdermal bandages, and the like. Included within the topical dosage forms are any lotion, stick, spray, ointment, paste, cream, gel, *etc.*, whether applied directly to the skin or via an intermediary such as a pad, patch or the like. Examples of dosage forms for suppository delivery include any solid or other dosage form to be inserted into a bodily orifice (particularly those inserted rectally, vaginally and urethrally). Examples of dosage units for transmucosal delivery include

depositories, solutions for enemas, pessaries, tampons, creams, gels, pastes, foams, nebulised solutions, powders and similar formulations containing in addition to the active ingredients such carriers as are known in the art to be appropriate. Examples of dosage units for depot administration include pellets or small cylinders of active agent or solid forms wherein the active agent is entrapped in a matrix of biodegradable polymers, microemulsions, liposomes or is microencapsulated. Examples of implantable infusion devices include any solid form in which the active agent is encapsulated within or dispersed throughout a biodegradable polymer or synthetic, polymer such as silicone, silicone rubber, silastic or similar polymer. Alternatively dosage forms for infusion devices may employ liposome delivery systems.

Depending on the disease to be treated and the subject's condition, the compounds of the present invention may be administered by oral, parenteral (for example, intramuscular, intraperitoneal, intravenous, ICV, intracisternal injection or infusion, subcutaneous injection, or implant), by inhalation spray, nasal, vaginal, rectal, sublingual, or topical routes of administration and may be formulated, alone or together, in suitable dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvants and vehicles appropriate for each route of administration. The pharmaceutical composition and method of the present invention may further comprise other therapeutically active compounds as noted herein which are usually applied in the treatment of the above mentioned conditions.

In the treatment or prevention of conditions which require copper modulation an appropriate dosage level will generally be about 0.001 to 100 mg per kg patient body weight per day which can be administered in single or multiple doses.

Preferably, the dosage level will be about 0.01 to about 25 mg/kg per day; more preferably about 0.05 to about 10 mg/kg per day. A suitable dosage level may be about 0.01 to 25 mg/kg per day, about 0.05 to 10 mg/kg per day, or about 0.1 to 5 mg/kg per day. Within this range the dosage may be about 0.005 to about 0.05, 0.05 to 0.5 or 0.5 to 5 mg/kg per day. For oral administration, the compositions are preferably provided in the form of tablets containing about 1 to 1000 milligrams of the active ingredient, particularly about 1, 5, 10, 15, 20, 25, 50, 75, 100, 150, 200, 250, 300, 400, 500, 600, 750, 800, 900, and 1000 milligrams of the active ingredient for the symptomatic adjustment of the dosage to the patient to be treated. The compounds may be administered on a regimen of 1 to 4 times per day, preferably once or twice per day.

It will be understood, however, that the specific dose level and frequency of dosage for any particular patient may be varied and will depend upon a variety of factors including the activity of the specific compound employed, the metabolic stability and length of action of that compound, the age, body weight, general health, sex, diet, mode and time of administration, rate of excretion, drug combination, the severity of the particular condition, and the host undergoing therapy.

The compounds of the present invention can be combined with other compounds having related utilities to prevent and treat tissue damage or excess tissue copper.

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All patents, publications, scientific articles, web sites, and other documents and materials referenced or mentioned herein are indicative of the levels of skill of those skilled in the art to which the invention pertains, and each such referenced

document and material is hereby incorporated by reference to the same extent as if it had been incorporated by reference in its entirety individually or set forth herein in its entirety. Applicants reserve the right to physically incorporate into this specification any and all materials and information from any such patents, publications, scientific articles, web sites, electronically available information, and other referenced materials or documents.

The specific methods and compositions described herein are representative of preferred embodiments and are exemplary and not intended as limitations on the scope of the invention. Other objects, aspects, and embodiments will occur to those skilled in the art upon consideration of this specification, and are encompassed within the spirit of the invention as defined by the scope of the claims. It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention. The invention illustratively described herein suitably may be practiced in the absence of any element or elements, or limitation or limitations, which is not specifically disclosed herein as essential. Thus, for example, in each instance herein, in embodiments or examples of the present invention, any of the terms "comprising", "consisting essentially of", and "consisting of" may be replaced with either of the other two terms in the specification. As used herein the term "and/or" means both "and" and "or". As used herein the addition of "(s)" as part of a word embraced both the singular and plural of that word. Also, the terms "comprising", "including", containing", *etc.* are to be read expansively and without limitation. The methods and processes illustratively described herein suitably may be

practiced in differing orders of steps, and that they are not necessarily restricted to the orders of steps indicated herein or in the claims. It is also that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise. Under no circumstances may the patent be interpreted to be limited to the specific examples or embodiments or methods specifically disclosed herein. Under no circumstances may the patent be interpreted to be limited by any statement made by any Examiner or any other official or employee of the Patent and Trademark Office unless such statement is specifically and without qualification or reservation expressly adopted in a responsive writing by Applicants.

The terms and expressions that have been employed are used as terms of description and not of limitation, and there is no intent in the use of such terms and expressions to exclude any equivalent of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention as claimed. Thus, it will be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

Other embodiments are within the following claims. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

WHAT WE CLAIM IS:

1. A method of treating tissue damage in a mammal selected from the myocardial tissue, tissue(s) of the vascular tree, and tissue(s) of organs dependent on the vascular tree, comprising subjecting said mammal to, and/or administering to said mammal, an amount of an agent or agents effective to lower the copper values content of said tissue(s) and treat said tissue damage.

2. A method of claim 1 wherein the patient is a human.

3. A method of claim 1 or 2 wherein the patient is not suffering from Wilson's Disease and has an elevated copper values content.

4. A method of claim 3 wherein there is at least one copper values status determination.

5. A method of claim 2 wherein the agent is trientine or a trientine type copper chelation agent.

6. A method of claim 2 wherein the agent is trientine or a trientine type copper chelation agent and the tissue copper values are lowered.

7. A method of claim 4 wherein trientine hydrochloride is administered at dosages or a dosage to provide, if parenteral, at least about 120 mg/day in a human patient, and if oral, at least about 600 to at least about 1200 mg/day in a human patient.

8. A method of claim 1 wherein the patient is a human being suffering from type 2 diabetes mellitus.

9. A method of claim 1 wherein improvement of the tissue repair arises from a restoration of, or substantial restoration, of normal tissue stem cell responses.

10. A method of claim 1 wherein the agent(s) is (are) selected from the group consisting of:

trientine (triene),

ethylenediaminetetraacetic acid (EDTA),

diethylenetriaminetetraacetic acid (DPTA),

2,2,2 tetramine tetrahydrochloride (TETA),

2,3,2 tetramine tetrahydrochloride,

D-penicillamine (DPA)

1,4,8,11 tetraazacyclotretradecane (Cyclam),

5,7,7',12,14,14' hexamethyl-1,4,8,11 tetraazacyclotretradecane (Cyclam S),

Sodium 2,3 dimercaptopropane-1-sulfonate (DMPS),

N-acetylpenicillamine (NAPA),

D-Penicillamine (PA),'

Desferroxamine,

2,3-dimercaptopropanol (BAL),

2,3-dimercaptosuccinic acid (DMSA),

trithiomolybdate,

3-7-Diazanonan-1,9-diamin (BE 6184),

1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid,

1,4,8,11-tetraazabicyclo[6.6.2]hexadecane,

4,11-bis(N,N-diethyl-amidomethyl)-1,4,8.11-tetraazabicyclo[6.6.2]hexadecane,

4,11-bis(amidoethyl)-1,4,8,11-tetraazabicyclo[6.6.2]hexadecane

clioquinol,

cuprizone,

N,N'-diethyldithiocarbamate,

zinc acetate,

zinc salts,

bathocuproinedisulfonic acid; bathocuprinedisulfonate,

neocuproine (2,9-dimethyl-1, 10-phenanthroline),

tetrathiomolybdate,

trimetazidine,

triethylene tetramine tetrahydrochloride,

2,3,2-tetraamine,

pyridine-2,6-bis(thiocarboxylic acid) or pyrrolidine dithiocarbamate,

tetraethylenepentamine,

N,N,N',N-tetrakis(2-pyridylemethyl) ethylenediamine

1,4,7,11-tetraazaundecane tetrahydrochloride,

tetraethylenepentamine pentahydrochloride,

D-Penicillamine (DPA),

1,10-orthophenanthroline,

3,4-Dihydroxybenzoic acid,

2,2'-bicinchinonic acid,

diamsar,

3, 4', 5, trihydroxystilbene (resveratrol),

mercaptodextran,

o-phenanthroline,

disulfiram (antabuse),

sar,

calcium trisodium diethylenetriaminepentaacetate (salt of cpd above),

and methimazole (1-methyl-2-thiolimidazole).

9. A method of claim 1 wherein the agent (agents) is (are) a zinc salt (zinc salts).

10. A method of claim 1 wherein said damage is from any one or more of (i) disorders of the heart muscle (cardiomyopathy or myocarditis) such as idiopathic cardiomyopathy, metabolic cardiomyopathy which includes diabetic cardiomyopathy, alcoholic cardiomyopathy, drug-induced cardiomyopathy, ischemic cardiomyopathy, and hypertensive cardiomyopathy, (ii) atheromatous disorders of the major blood vessels (macrovascular disease) such as the aorta, the coronary arteries, the carotid arteries, the cerebrovascular arteries, the renal arteries, the iliac arteries, the femoral arteries, and the popliteal arteries, (iii) toxic, drug-induced, and metabolic (including hypertensive and/or diabetic disorders of small blood vessels (microvascular disease) such as the retinal arterioles, the glomerular arterioles, the vasa nervorum, cardiac arterioles, and associated capillary beds of the eye, the kidney, the heart, and

the central and peripheral nervous systems, (iv) plaque rupture of atheromatous lesions of major blood vessels such as the aorta, the coronary arteries, the carotid arteries, the cerebrovascular arteries, the renal arteries, the iliac arteries, the fermoral arteries and the popliteal arteries.

11. A method of claim 1 wherein the patient is suffering from and/or is predisposed to heart failure.

12. A method of claim 11 wherein the patient is suffering from type 2 diabetes mellitus.

13. The use of a compound (a) which itself *in vivo* or (b) which has at least one metabolite *in vivo* that is (i) a copper chelator or (ii) otherwise reduces available copper values for the production of a pharmaceutical composition or dosage unit able to reduce the level of copper in a mammal thereby to elicit by a lowering of copper values in a mammalian patient an improvement of tissue repair of damaged tissue selected from that of the myocardium, the vascular tree and organs dependent on the vascular tree.

14. The use of claim 13 wherein the damage is that which has arisen from a disease selected from the group consisting of (i) disorders of the heart muscle (cardiomyopathy or myocarditis) such as idiopathic cardiomyopathy, metabolic cardiomyopathy which includes diabetic cardiomyopathy, alcoholic cardiomyopathy, drug-induced cardiomyopathy, ischemic cardiomyopathy, and hypertensive cardiomyopathy, (ii) atheromatous disorders of the major blood vessels (macrovascular disease) such as the aorta, the coronary arteries, the carotid arteries, the cerebrovascular arteries, the renal arteries, the iliac arteries, the femoral arteries, and the popliteal

arteries, (iii) toxic, drug-induced, and metabolic (including hypertensive and/or diabetic disorders of small blood vessels (microvascular disease) such as the retinal arterioles, the glomerular arterioles, the vasa nervorum, cardiac arterioles, and associated capillary beds of the eye, the kidney, the heart, and the central and peripheral nervous systems, (iv) plaque rupture of atheromatous lesions of major blood vessels such as the aorta, the coronary arteries, the carotid arteries, the cerebrovascular arteries, the renal arteries, the iliac arteries, the fermoral arteries and the popliteal arteries.

15. The use of claim 13 or 14 wherein the compound is selected from the group consisting of:

trientine (triene),

ethylenediaminetetraacetic acid (EDTA),

diethylenetriaminetetraacetic acid (DPTA),

2,2,2 tetramine tetrahydrochloride (TETA),

2,3,2 tetramine tetrahydrochloride,

D-penicillamine (DPA)

1,4,8,11 tetraazacyclotretradecane (Cyclam),

5,7,7',12,14,14' hexamethyl-1,4,8,11 tetraazacyclotretradecane (Cyclam S),

Sodium 2,3 dimercaptopropane-1-sulfonate (DMPS),

N-acetylpenicillamine (NAPA),

D-Penicillamine (PA),'

Desferroxamine,

2,3-dimercaptopropanol (BAL),

2,3-dimercaptosuccinic acid (DMSA),

trithiomolybdate,

3-7-Diazanonan-1,9-diamin (BE 6184),

1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid,

1,4,8,11-tetraazabicyclo[6.6.2]hexadecane,

4,11-bis(N,N-diethyl-amidomethyl)-1,4,8.11-tetraazabicyclo[6.6.2]hexadecane,

4,11-bis(amidoethyl)-1,4,8,11-tetraazabicyclo[6.6.2]hexadecane

melatonin,

clioquinol,

cuprizone,

N,N'-diethyldithiocarbamate,

zinc acetate,

zinc salts,

bathocuproinedisulfonic acid; bathocuprinedisulfonate,

neocuproine (2,9-dimethyl-1, 10-phenanthroline),

tetrathiomolybdate,

trimetazidine,

triethylene tetramine tetrahydrochloride,

2,3,2-tetraamine,

pyridine-2,6-bis(thiocarboxylic acid) or pyrrolidine dithiocarbamate,

tetraethylenepentamine,

N,N,N',N-tetrakis(2-pyridylemethyl) ethylenediamine

1,4,7,11-tetraazaundecane tetrahydrochloride,

tetraethylenepentamine pentahydrochloride,

D-Penicillamine (DPA),

1,10-orthophenanthroline,

3,4-Dihydroxybenzoic acid,

2,2'-bicinchinonic acid,

diamsar,

3, 4', 5, trihydroxystilbene (resveratrol),

mercaptodextran,

o-phenanthroline,

disulfiram (antabuse),

sar,

calcium trisodium diethylenetriaminepentaacetate (salt of cpd above),

and methimazole (1-methyl-2-thiolimidazole).

16. The use of claim 13 wherein the compound is trientine or a trientine-type copper chelation agent.

17. The use of claim 13 which includes one or more pharmaceutically acceptable excipients, diluents and/or carriers.

18. A dosage unit resulting from the use of claim 13.

19. A method of treating a mammalian subject at risk of developing, with suspected or with actual tissue disease to the myocardium, the vascular tree and/or organs dependent on the vascular tree, which method comprises the step of

administering to the subject one or more agents capable of decreasing the copper values content, whereby tissue repair in said subject is enhanced.

20. A method of claim 19 wherein said subject is not suffering from Wilson's Disease and has an elevated copper values content.

21. The method of claim 19 wherein said subject is a human.

22. A method of any of claims 19, 20 or 21 wherein the agent(s) is (are) a chelator (chelators) of copper.

23. A method of any of claims 19, 20 or 21 wherein the agent(s) has (have) an affinity for copper over that of iron.

24. A method of treating a mammalian subject at risk of developing, with suspected or with actual tissue disease to the myocardium, the vascular tree and/or organs dependent on the vascular tree, which method comprises or includes the steps of:

determining the copper status of the subject, and

if the copper status of a subject is elevated yet the patient is not suffering from Wilson's Disease, subjecting the patient to and/or administering to the subject one or more agents capable of decreasing the subject's copper values content, whereby tissue repair in said subject is enhanced.

25. A method of claim 23 which involves continual evaluating or monitoring of the copper status of the subject.

26. A method of claim 23 or 24 wherein the determination of the copper status is by reference to extra cellular copper values.

27. A method of claim 23 wherein the decreasing of the subject's copper values content is from an elevated status being that typical of the copper values status of a human patient suffering from type 2 diabetic mellitus over that of a non-diabetic.

28. A method of claim 23 which includes the step of diagnosing and/or evaluating or monitoring hypertension.

29. A method of claim 23 which includes the step of diagnosing alcoholism.

30. A method of claim 23 which includes the step of diagnosing and/or evaluating or monitoring a glucose mechanism abnormality of the patient.

31. A method of claim 29 wherein the abnormality is type 2 diabetes mellitus, impaired glucose tolerance and/or impaired fasting glucose.

32. A method of claim 23 which includes the step of diagnosing and/or evaluating or monitoring macrovascular, microvascular, toxic and/or metabolic damage in the patient.

32. A method of claim 23 wherein the damage is that of any one or more of:

(i) disorders of the heart muscle (cardiomyopathy or myocarditis) such as idiopathic cardiomyopathy, metabolic cardiomyopathy which includes diabetic cardiomyopathy, alcoholic cardiomyopathy, drug-induced cardiomyopathy, ischemic cardiomyopathy, and hypertensive cardiomyopathy,

or

(ii) atheromatous disorders of the major blood vessels (macrovascular disease)

such as the aorta, the coronary arteries, the carotid arteries, the cerebrovascular arteries, the renal arteries, the iliac arteries, the femoral arteries, and the popliteal arteries,

or

(iii) toxic, drug-induced, and metabolic (including hypertensive and/or diabetic disorders of small blood vessels (microvascular disease) such as the retinal arterioles, the glomerular arterioles, the vasa nervorum, cardiac arterioles, and associated capillary beds of the eye, the kidney, the heart, and the central and peripheral nervous systems,

or

(iv) plaque rupture of atheromatous lesions of major blood vessels such as the aorta, the coronary arteries, the carotid arteries, the cerebrovascular arteries, the renal arteries, the iliac arteries, the fermoral arteries and the popliteal arteries.